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**Resistance Mechanisms to Viruses in Plants
Associated with Antiviral Substances (Inhibitors
of Virus Replication)**

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TABLE OF CONTENTS

	<u>PAGE</u>
Abstract	I
Objectives	III
Body of Report	
A. Evaluation of IVR on different hosts	1
B. Definition of a "unit" and dose response of IVR	4
B-1. Effect of IVR on TMV replication measured by dot-spot hybridization	5a
C. Improving methods for preparation of IVR	
1. Improving protoplast production	6
2. Preparation of antisera and further purification of IVR by immunoaffinity chromatography	7
3. An IVR like compound from "green islands"	21
4. Obtaining IVR directly from plants	
a. Tobacco	48
b. From Pinto Bean	54
5. Preliminary attempts to purified IVR with HPLC.	60
D. Isolation of antiviral substances	62
E. Binding of IVR to Con-A	75
F. Production of IVR concurrently with resistance and its suppression by antimetabolites	77
G. Mode of Action of IVR	
1. Effect on plasmodesmata	78
2. Effect on protein synthesis	82
3. Effect of antimetabolites on IVR activity	83
H. Preformed inhibitors of virus infection	
1. From cotton	85
2. From <u>N. glutinosa</u>	87
3. From Datura	88

I.	UV photobiology of virus-host interaction	108
	Description of Cooperation	117
	Evaluation of Research Achievements	117
	List of Publications	121

ABSTRACT

The inhibitor of virus replication (IVR) obtained from the incubation medium of Samsun NN protoplasts inoculated with tobacco mosaic virus (TMV) was further studied: IVR was found to be effective when applied by spraying to cucumber and tomato plants, reducing cucumber mosaic virus (CMV) and TMV titers, respectively. Inhibition rates of IVR in protoplast and leaf disks was found to be dose responsive; and inhibition was evident also by dot spot hybridization.

IVR production was increased markedly, mainly by improving the protoplast technology. Using antisera prepared against the two biologically active fractions of IVR, it was shown that the two are serologically identical. The antisera were also used for further purification of IVR by immunoaffinity chromatography, and preliminary work was done to purify IVR using high performance liquid chromatography (HPLC). An IVR-like compound was found to be associated with another resistance mechanism "green islands" in tobaccos infected with CMV. IVR could also be obtained directly from the intercellular wash fluid of Samsun NN tobaccos inoculated with TMV, and an inhibitory compound was also obtained in Montana from the wash fluid of Pinto bean leaves. In parallel work in Montana the tobacco protoplast system was used to isolate both an induced (58,000 d) and an endogenous (24,000 d) inhibitor of virus infection. Both substances were proteinaceous, and evidence was obtained that the induced compound was a phosphoglycoprotein, whose activity involved inhibition of viral RNA-directed protein synthesis.

Actinomycin D and chloramphenicol suppressed IVR synthesis and concomittantly increased virus replication in resistant protoplasts.

Studies on the mode of action of IVR indicated that IVR does not effect plasmodesmata number or protein synthesis of the host.

Inhibitors of infection were found in cotton, N. glutinosa, and Datura, the latter acting by binding competitively to cell receptors and by altering their affinity for virus.

Some evidence was obtained that UV-C partially overcomes localized acquired resistance to TMV infection in hypersensitive host, and the resistance of "green islands" in a systemic host.

Although UV-C produced delocalization of TMV lesions in Pinto bean, it failed to do so in Nicotiana spp. However, irradiation 24 hrs before inoculation decreased both lesion numbers and area for both test species.

d. Objectives

Evaluating concentrated IVR preparations for control of several viruses in different intact plant species.

Improving methods for preparation of IVR from protoplasts, and attempting to isolate IVR or a similar agent from different types of resistant tissues especially from "green islands".

Studying the mode of induction of IVR also with non-viral polyanions in isolated protoplasts and intact plants, parallel to the development of resistance.

Further characterization of the chemical nature of IVR, preparation of an antiserum, definition of a unit of activity, and its mode of penetration(?) and action.

Testing new-host virus combinations for acquired resistance to virus infection with possible association of induced antiviral agents; and studying preformed virus inhibitors, especially those acting on early post-establishment stages of viral replication.

Evaluating the nature of the chemical signal of systemic acquired resistance with further work on ultraviolet photobiology of viral infection.

e. Body of Report

A. Evaluation of IVR on different additional virus-host combinations
(in Israel).

Previously it was shown that IVR applied by spraying to intact tobacco plants inhibited TMV (Gera & Loebenstein, 1983). In further experiments the effect of IVR applied by spraying to tomato plants inoculated with TMV and cucumber plants inoculated with cucumber mosaic virus (CMV) was evaluated.

Cucumber plants about 10-12 days after emergence were inoculated with CMV and sprayed after 5 hr with a solution containing 3 units of IVR in 10 ml of distilled water. At various times after inoculation two 11 mm disks were sampled, homogenized in 2 ml of phosphate-buffered saline (PBS) and their infectivity assayed on Vigna sinensis "Blackeye". For controls, similar plants were inoculated with CMV and either sprayed with mock-IVR (prepared from non-inoculated protoplasts - Loebenstein & Gera, 1981), or without spraying and assayed similarly for CMV. No differences in the infectivity assays between mock-IVR sprayed plants or non-sprayed plants were observed, while in the IVR sprayed plants a reduction of 60-77% in CMV titer was observed (average from 2 experiments) (Fig. 1).

In a parallel experiment tomato plants 10-12 days after transplanting (about 7 cm in height) were sprayed also with 3 units of IVR in 10 ml of distilled water, 5 hr after their inoculation with

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In a parallel experiment tomato plants 10-12 days after transplanting (about 7 cm in height) were sprayed after 5 hr with 3 units of IVR in 10 ml of distilled water, 5 hr after their inoculation with

TMV. Sampling of disks was as described before 2,3 and 4 days after inoculation. Assays of infectivity was on 12 half-leaves of Nicotiana glutinosa. IVR reduced infectivity by 64-73% (Fig. 2).

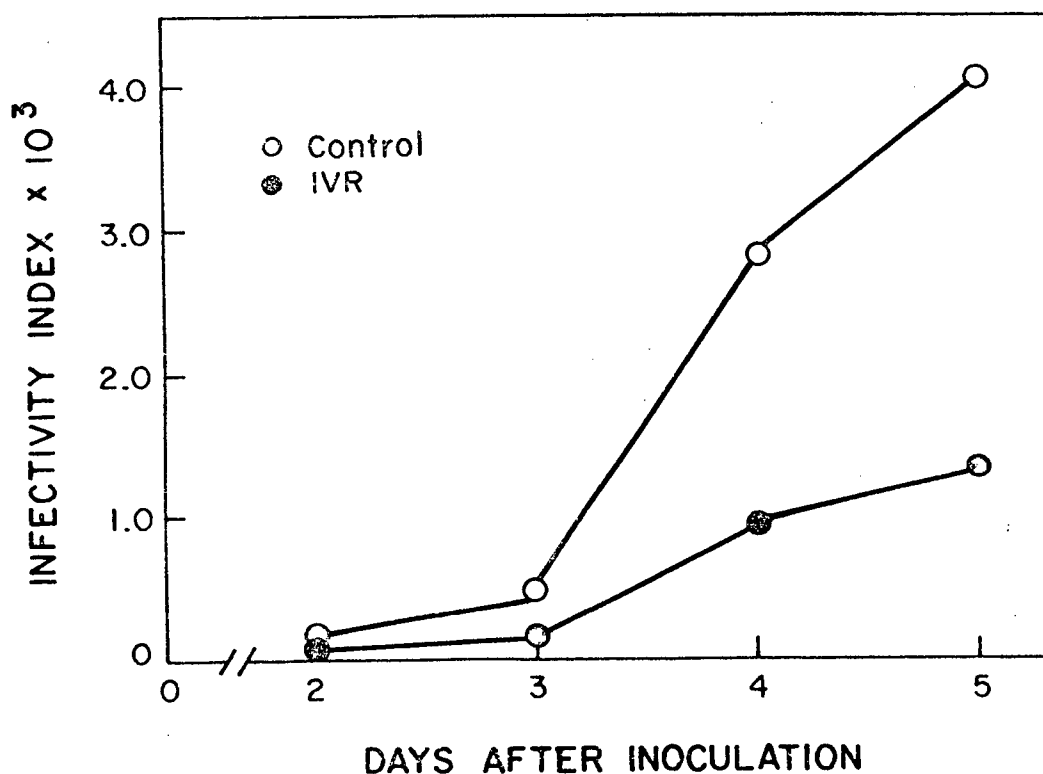


Fig. 1. CMV replication in cucumber plants sprayed once with 3 units of IVR or with mock-IVR (control). Infectivity index-average number of local lesions on one leaf of Vigna sinensis "Blackeye" multiplied by inoculum dilution factor; i.e. assay after 2 days inoculum not diluted - dilution factor = 1; after 3 and 4 days inoculum diluted 1:10 - dilution factor = 10; after 5 days inoculum diluted 1:100 - dilution factor = 100. Averages from 2 experiments.

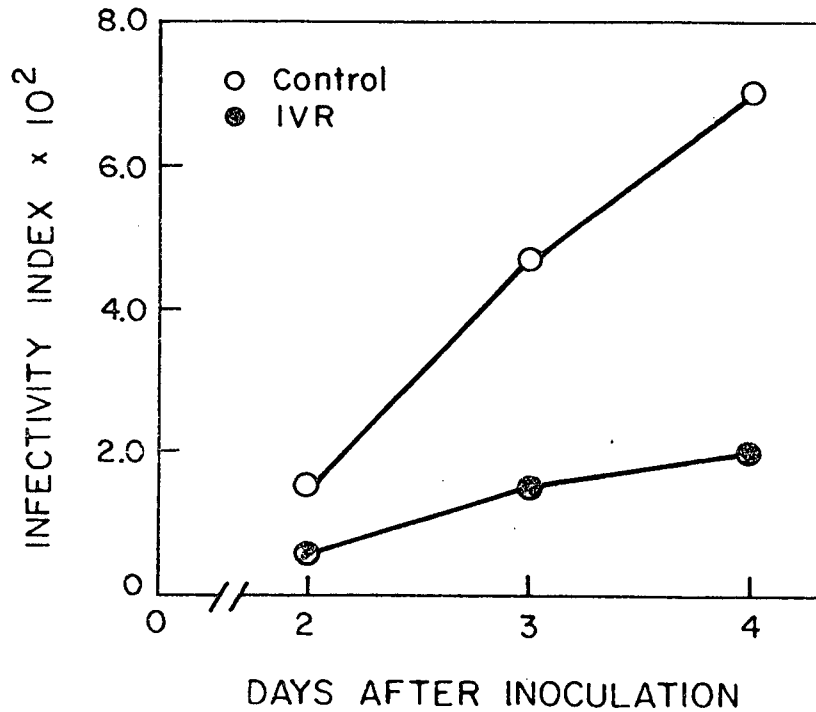


Fig. 2. TMV replication in tomato plants sprayed once with 3 units of IVR or with mock-IVR (control). Infectivity index-average number of local lesions on one half-leaf of *N. glutinosa* multiplied by inoculum dilution factor. Averages from 2 experiments.

B. Definition of a "unit" and dose response of IVR. (in Israel)

The amount of IVR obtained from 10^6 tobacco NN protoplasts, incubated for 72 hr after inoculation with TMV were termed "one unit".

As estimated by amino acid analysis after high pressure liquid chromatography (HPLC) and by staining reactions with Coomassie brilliant blue after polyacrylamide gel electrophoresis, 100 units of IVR (obtained from the incubation medium of 10^8 protoplasts) was equivalent to approx. 1 ug protein (in collaboration with Prof. Y. Burstein and Dr. V. Buchner, Weizmann Institute of Science, Rehovot).

The effect of increasing concentrations of IVR (after ZnAc_2 precipitation, Loebenstein & Gera, 1981) on inhibition of TMV replication was tested both with leaf disk (Gera & Loebenstein, 1983) and protoplast (Loebenstein & Gera, 1981) assays. Fig. 3 shows the level of inhibition obtained with increasing concentrations of IVR in both assays, as determined by the local lesion assay 72 hr after inoculation. Inhibition rates were dose responsive.

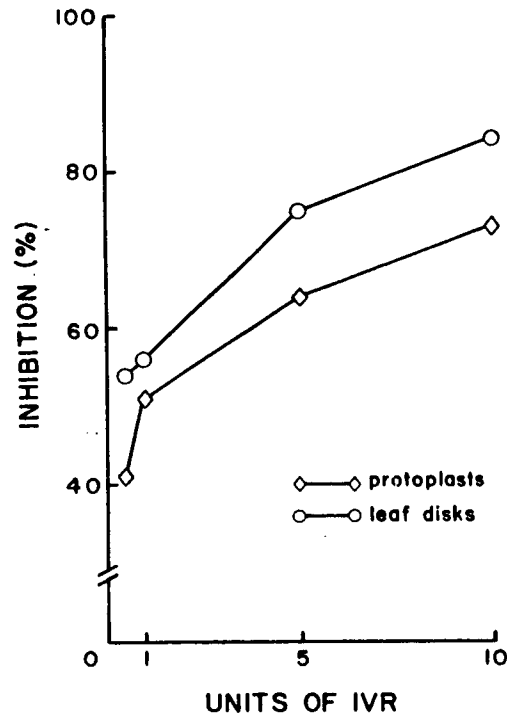


Fig. 3. Effect of increasing concentrations of IVR on inhibition of TMV replication in protoplasts and leaf disks of tobacco Samsun determined by local lesion assay on N. glutinosa. Averages from 3-5 experiments.

B-1 Effect of IVR on TMV replication as measured by dot-spot hybridization (in Israel)

TMV-infected protoplasts were treated with IVR and control preparation, 5 hrs after inoculation. 72 hrs after incubation the protoplasts were removed and frozen.

The preparation of RNA for hybridization was as follows:

Frozen protoplasts (10×10^6) were solubilized with 2.5 ml of an extraction buffer containing 25 mM Tris-HCl, pH-7.5, 25mM KCl, 25mM $MgCl_2$ and 1% SDS (Bourque et al., 1973) and were shaken vigorously with an equal volume of water-saturated phenol for 20 min at room temperature. The emulsion was broken by centrifugation and the aqueous phase was re-extracted with phenol. The aqueous phase was collected and 5M NaCl was added to a final concentration of 0.2M. RNA was precipitated by the addition of 2.5 volume of cold ethanol at $-20^{\circ}C$.

The precipitate was dissolved in deionized water and 3 ul of each sample was then spotted on a nitrocellulose paper that was presoaked in 20 x SSC.

The cDNA done was a gift from Dr. Dawson (Riverside, California). Labelling of cDNA with [^{32}P] was carried out by nick-translation (Maniatis et al., 1975). The probe was heated to $100^{\circ}C$ for 5 min and then cooled on ice before being added to the RNA spotted on the nitrocellulose paper.

Hybridization was carried out at 42°C in a buffer containing 50% formamide, 4 x SSC 1 x Denhardt and 50 mM Na-Phosphate buffer, pH 6.5, essentially as described by Thomas (1980).

Fig 3-1 illustrates the hybridization experiments with RNA isolated from healthy nontreated protoplasts, infected-IVR-treated, infected-control-treated, infected-non-treated-protoplasts, compared to 60ng TMV-RNA.

These results indicate that in IVR-treated protoplasts indeed less TMV-RNA is produced concomittant with the effect on extractable infectivity.

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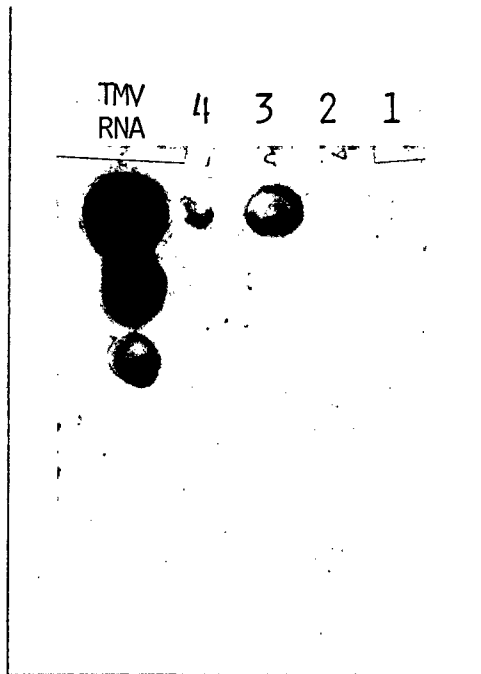


Fig. 3-1 Autoradiography of dot-blot of TMV-RNA from healthy non-treated protoplasts (1); TMV inoculated IVR-treated (2); TMV-inoculated control-treated (3), TMV-inoculated non-treated protoplasts (4) and TMV-RNA using a ^{32}P cDNA clone to TMV-RNA.

C. Improving Methods for Preparation of IVR

1. Improving production of protoplasts. (in Israel)

Production of protoplasts was markedly improved from about 5×10^6 protoplasts per 120 cm petri dish to about $11-13 \times 10^6$ per dish. Various enzyme concentrations, time of incubation with enzymes and keeping the tobacco plants at various temperatures before sampling them for protoplast production were evaluated. As shown in Table 1, procedure 2 (shortening of incubation time with enzymes) and procedure 3 (transferring tobacco plants 7-10 days before sampling leaves to a $22 \pm 2^\circ\text{C}$ constant temperature chamber) were found to double protoplast yield compared to the original procedure (procedure 1).

Procedure 3 gives a regular and stable yield of protoplasts whereby 2 technicians produce regularly 300×10^6 protoplasts per week giving 300 units of IVR.

Table 1. Yields of protoplasts from tobacco NN by the three procedures.

Procedure	Temp. in green house	Enzyme conc. (%)		Incubation with enzymes(hrs)	Protoplast yield (10^6 protoplasts petri dish)
		Cellulase	Macerozyme		
1 ^a	15-30°C	0.15	0.015	16.5	5-6
2 ^a	15-30°C	0.4	0.04	12	11-13
3 ^b	22 ± 2°C	0.3	0.03	16	10-13

^a Plants grown in greenhouse without temperature control.

^b Plants transferred 7-10 days before sampling for protoplast production to a 22 ± 2°C greenhouse chamber.

2. Preparation of antisera to IVR and preliminary attempts for additional IVR purification by immunoaffinity chromatography (in Israel).

INTRODUCTION

Previously, we reported that a substance(s) inhibiting virus replication (IVR) is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a cultivar in which the infection in the intact plant is localized. IVR inhibited virus replication in protoplasts from both local lesion-responding resistant (NN) and systemic-responding susceptible (Samsun) plants (Loebenstein & Gera, 1981). In further studies it was found that IVR inhibited virus

replication also in leaf tissue disks and in intact leaves when applied to cut stems or by spray. IVR was partially purified using ZnAc_2 precipitation and yielded two biologically active principles with molecular weights of approximately 26,000 (IVR-1) and 57,000 (IVR-2), as determined by gel filtration. IVR was found to be sensitive to the proteolytic enzymes trypsin and chymotrypsin, but not to RNase, suggesting that IVR has a proteinaceous nature (Gera & Loebenstein, 1983).

Here we report on (a) the preparation of an antiserum to IVR and to its two biologically active principles, (b) their evaluation also against Sela's antiviral factor (AVF) (Sela et al., 1966; Mozes et al., 1978; Sela, 1981) and interferon, and (c) use of the antiserum in immunoaffinity chromatography of IVR.

MATERIALS AND METHODS

Preparation of protoplasts and IVR. Growing of Nicotiana tabacum L. cv. Samsun NN plants and the preparation of protoplasts and IVR by precipitation with ZnAc_2 were as described previously (Loebenstein & Gera, 1981). Control preparations were obtained similarly, from sham-inoculated protoplasts.

A lyophilized ZnAc_2 preparation obtained from 3×10^7 inoculated protoplasts was dissolved in 1-2 ml of 0.1 M phosphate buffer, pH 6.0, placed on a Sephadex G-75 (Superfine, Pharmacia) column, and eluted with the same buffer. Fractions of molecular weight equivalent to 26,000 (IVR-1) and 57,000 daltons (IVR-2) were collected (Loebenstein & Gera, 1981), lyophilized, dissolved in 1 ml 0.05 M phosphate buffer,

pH 7.0, and dialysed against the same buffer. The two fractions were either mixed or used separately for injection of rabbits.

Preparation of antisera. One ml of purified IVR (separate or mixed fractions) was emulsified with an equal volume of Freund's incomplete adjuvant. Six intramuscular injections, each with 30 units of IVR (isolated from the incubation medium of 30×10^6 protoplasts), were given to one rabbit. The first four were given at intervals of 4 days and the last two injections were administered 3 weeks later, again at a 4-day interval. One month later, one intravenous injection (without adjuvant) was given. The rabbits were bled several days after the last injection and the serum was stored in an equal volume of glycerol at -20°C (non-absorbed antiserum).

To eliminate nonspecific antibodies, 1-2 ml of the antisera was mixed with 50×10^6 sham-inoculated protoplasts. After 6 h, the protoplasts were removed by centrifugation.

Enzyme-linked immunosorbent assay (ELISA) and agar-gel-diffusion tests. For purification of γ -globulin, conjugation of alkaline phosphatase with γ -globulin- the method described by Clark & Adams (1977)- was followed. The γ -globulins used for ELISA were further absorbed with a lyophilized control preparation obtained from the incubation medium of 50×10^6 mock-inoculated protoplasts.

Agar-gel-diffusion tests were done in 55 mm petri dishes containing a 4 mm layer (9 ml) of 0.75% agar (Bacto-Agar, Difco), 0.001 M EDTA, 0.85% NaCl and 0.2% sodium azide at pH 7.8 (Tomlinson et al., 1973). Agar plates were incubated in a moist chamber at 22°C for 15 h.

Neutralization of IVR biological activity by specific antiserum.

Polystyrene microplates were coated with γ -globulin specific to IVR (0.02 mg/ml). The plates were incubated for 4 h at 37°C, and washed with phosphate buffered saline containing 0.05% Tween 20. Then 0.2 ml of purified IVR (3 units) was added to triplicate wells. After 3 h of incubation the fluid from the wells was collected and its biological activity tested on TMV-infected tobacco protoplasts (Loebenstein & Gera, 1981). For controls, IVR placed on wells coated with γ -globulin from non-immunized rabbits was used.

Immunoaffinity chromatography. IgGs were purified from the mixed antiserum using saturated ammonium sulfate and a DEAE-cellulose column. Purified IgGs were coupled to activated Sepharose 4B (Pharmacia). Activation of Sepharose was done with sodium borohydride (Charbonneau & Cormier, 1979). The Sepharose was washed with deionized water on a glass filter and suction-dried before use. Ten g of the dried Sepharose was then suspended in 10 ml of 6N NaOH containing 30 mg sodium borohydride and 10 ml diglycidyl ether. The mixture was gently stirred for 8 h at room temperature, and the gel was then washed with 1 liter of deionized water and dried. Coupling was done immediately after the washing according to Livingston (1974). Dry activated Sepharose was mixed with IgG dissolved in 0.1 M sodium bicarbonate, pH 8, at 1:200 or 1:1000 (W/V) and left for 18 h at 4°C. After that the suspension was centrifuged at low speed. To determine the amount of IgG coupled to the Sepharose, the absorbency at 280nm in the supernatant fluid was measured and compared with the absorbency of

the initial uncoupled IgG solution. About 90-95% of the IgG was bound to the Sepharose. Columns of either 3 ml or 1 ml volume were prepared and washed with 0.1M carbonic buffer until no absorbency at 280 nm was detected.

Lyophilized IVR (40-45 units obtained from the incubation medium of $40-45 \times 10^6$ protoplasts) after ZnAc_2 precipitation was dissolved in 0.5 ml of 0.1M phosphate buffer, pH 7, and placed on the column. Washings and elutions were performed as follows:

1. Washing with 10 volumes (of respective column) of 0.1 M phosphate buffer, pH 7, containing 0.1 M NaCl. All the eluate was collected.
2. Elution with 2-3 volumes of 0.1 M glycine buffer pH 6, containing 1 M NaCl. Three fractions of 1.5-2 ml each were collected.
3. Elution with 2-3 volumes of 1M glycine buffer pH 2.6. Four fractions of 1.5-2 ml each were collected.
4. Washing with 5-10 volumes of 0.1 M phosphate buffer, pH 7, containing 0.14 M NaCl. All the eluate was collected.

A control preparation, obtained from a similar number of mock-inoculated protoplasts, was passed through the same column, eluted, and collected in a similar way.

All fractions were dialyzed four or five times, against 4 liters of deionized water, after which they were freeze-dried and dissolved in 1.5 ml deionized water, and the biological activity tested on TMV-infected tobacco protoplasts (Loebenstein & Gera, 1981), in comparison with the respective control fractions.

RESULTS

Serological evaluation of IVR antiserum

The mixed non-absorbed IVR antiserum enabled a clear distinction in ELISA between ZnAc_2 preparations from incubation media of TMV-infected protoplasts and those obtained from mock-inoculated protoplasts. Thus, ZnAc_2 preparations from 1, 5 and 10 million infected protoplasts per well gave E_{405} values of 0.316, 0.562 and 0.800, respectively (averages of two experiments, each on three wells), compared with 0.109, 0.109 and 0.116 when ZnAc_2 preparations from comparable numbers of mock-inoculated protoplasts were assayed. When an adsorbed mixed antiserum was used, ZnAc_2 preparations from 1, 3 and 5 million protoplasts per well gave respective E_{405} values of 0.38, 0.58 and 0.73 (averages from three experiments, each on three wells), compared with 0.035, 0.04 and 0.065 from respective numbers of control protoplasts.

The mixed non-absorbed IVR antiserum did not react in ELISA with purified TMV, TMV-coat protein or TMV-infected-Samsun tobacco leaf extracts.

Results on the activity of absorbed antisera prepared against the 26,000 (IVR-1) and the 57,000 (IVR-2) fractions separately, are summarized in Table 2. As seen with ELISA, no differences were observed when each of the two antisera was tested against these two IVR fractions, indicating that the two fractions serologically do not differ markedly.

In agar-gel diffusion tests clear precipitation lines were obtained between IVR (10 units after ZnAc_2 precipitation dissolved in

0.5 ml 0.1M phosphate buffer, pH 7), purified IVR-1, IVR2 and the mixed absorbed IVR antiserum (diluted 1:5). The precipitation lines fused without spurs (Fig. 4a). Similar results were obtained when absorbed antisera to IVR-1 and IVR-2 were used. The two antisera reacted with both fractions, giving precipitation lines that fused without spurs (Fig. 4b,c), thus indicating that the fractions are serologically close.

No lines were observed between the antisera and the respective control preparations, *i.e.*, ZnAc_2 preparation from the incubation medium of mock-inoculated protoplasts and the parallel control fractions to IVR-1, IVR-2 (Fig. 4a,b,c).

Neutralization of IVR's biological activity by specific antiserum

The mixed non-absorbed IVR antiserum totally abolished the inhibitory activity of IVR, while with normal antiserum no effect on the biological activity of IVR was observed (Table 3).

Evaluation of IVR antiserum with AVF and interferon

To determine whether IVR has similarities to AVF (Sela *et al.*, 1966; Mozes *et al.*, 1978), and to human interferon, the IVR antisera were tested by the double-gel-diffusion test against AVF (obtained from Dr. I. Sela) and human leukocyte interferon (gift from National Institutes of Health, Bethesda, MD, U.S.A.).

No precipitation lines were obtained either with 10^5 units of human leukocyte interferon or with 10 ug AVF per well when the mixed absorbed antiserum or the absorbed antisera to IVR-1 or IVR-2 were used.

Binding and elution of IVR by immunoaffinity chromatography

Results of two separate experiments are given in Table 4. Columns 1 was of 3 ml in volume, containing 2.5g activated Sepharose coupled to 2.5 mg mixed non-absorbed IVR-IgG. Column 2 was 1 ml in volume, containing 1 g of activated Sepharose coupled to 5 mg IgG.

Peaks of activity were eluted at two positions, one after elution with 0.1 M phosphate buffer, pH 6, containing 1M NaCl (fractions 2 and 3), and the second after elutions with 1 M glycine, pH 2.6 (fractions 6 and 7).

These results indicate that IVR can be bound and released by immunoaffinity chromatography.

DISCUSSION

Antisera to IVR were obtained by injecting relatively large amounts of IVR: 180 "units" obtained from 180×10^6 protoplasts incubation medium. This was expected, as both the 26,000 and the 57,000 fractions with biological activity occurred at positions where no OD₂₈₀ peaks were observed, thus suggesting that IVR is active at extremely low concentrations (Loebenstein & Gera, 1981).

Antisera prepared against the two main biologically active fractions, IVR-1 (26,000) and IVR-2 (57,000), were highly cross-reactive, and precipitation lines fused completely, indicating the presence of identical determinants in IVR-1 and IVR-2. This supports the previous suggestion (Loebenstein & Gera, 1981) that the 57,000 fraction is a dimer of the 26,000 fraction.

The IVR antiserum gave no reaction with TMV, TMV coat protein, AVF or human leukocyte interferon, pointing up the difference between IVR and these fractions. The antiserum abolished IVR's biological activity and could be used in immunoaffinity chromatography. Elution of the biological activity occurred in two zones. This may have been due to the presence of several antigenic determinants on the IVR molecule. The efficiency of using immunoaffinity chromatography for additional purification of IVR will be evaluated in the future.

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TABLE 2

ELISA EVALUATION OF ANTISERA AGAINST IVR-1 (26,000) AND IVR-2
(57,000)¹

<u>Fraction</u>	<u>E₄₀₅²</u> <u>IVR-1 antiserum</u>	<u>IVR-2 antiserum</u>
IVR-1	0.680 ± 0.032	0.665 ± 0.028
IVR-2	0.630 ± 0.051	0.675 ± 0.054
Control ³	0.085 ± 0.023	0.063 ± 0.028

¹ Three units each of IVR-1 and IVR-2 per well.

² Averages from three experiments (three replicates each), ± standard error.

³ Control preparations from mock-inoculated protoplasts.

TABLE 3
NEUTRALIZATION OF IVR'S BIOLOGICAL ACTIVITY BY SPECIFIC ANTISERUM¹

Preparation ²	Wells coated with γ -globulin of	Infectivity from test protoplasts treated with the respective preparation ³	Percentage inhibition, IVR/control
IVR	- ⁴	9.9 \pm 1.69	
Control	- ⁴	28 \pm 2.28	65
IVR	Normal	10.2 \pm 1.84	
Control	Normal	29.4 \pm 1.77	65
IVR	anti-IVR	25.7 \pm 2.76	
Control	anti-IVR	26.2 \pm 2.75	2
Control protoplasts ⁵		28 \pm 3.25	

¹ Antiserum (non-adsorbed) prepared by injecting the two IVR fractions (mixed). Averages of two experiments.

² ZnAc₂ preparations from incubation medium of 3x10⁶ infected or non-infected control protoplasts per well.

³ Average number of local lesions and standard error per 10⁶ Samsun NN protoplasts 72 h after inoculation, on one half-leaf of Nicotiana glutinosa calibrated to standard TMV (0.2 ug/ml), which yielded 60 lesions per half-leaf.

⁴ Non-coated wells.

⁵ Infectivity from inoculated non-treated protoplasts.

TABLE 4
IMMUNOAFFINITY CHROMATOGRAPHY OF IVR

		Column 1 ^a		Column 2 ^b			
		Infectivity ^c		Infectivity ^c			
Elution buffer	Fraction No.	IVR	Control medium ^d Percent inhibition of infectivity	IVR	Control medium ^d Percent inhibition of infectivity		
0.1M phosphate + 0.15M NaCl pH 7	1	27.9 ± 6.2	19.8 ± 4.3	0	17.5 ± 3.9	8.1 ± 5.5	0
0.1M phosphate + 1M NaCl pH 6	2	12.0 ± 3.9	25.7 ± 6.5	53	4.3 ± 2.4	15.2 ± 2.1	72
	3	7.4 ± 2.4	15.5 ± 4.5	52	15.2 ± 2.2	16.2 ± 4.3	0
	4	33.4 ± 6.0	35.3 ± 4.7	0	25.2 ± 1.9	27.8 ± 2.1	0
1M glycine pH 2.6	5	15.7 ± 5.3	20.5 ± 3.7	23	13.3 ± 2.7	12.9 ± 1.8	0
	6	7.9 ± 2.5	18.6 ± 3.7	58	5.8 ± 1.5	16.2 ± 2.3	65
	7	18.4 ± 5.0	22.1 ± 4.7	17	9.0 ± 0.1	19.0 ± 2.1	53
	8	31.2 ± 8.0	21.8 ± 8.7	0	10.2 ± 2.1	8.5 ± 0.3	0
0.1 phosphate + 0.15 NaCl pH 7	9	27.9 ± 6.2	19.8 ± 4.3	0	8.3 ± 3.1	9.5 ± 1.2	0
Control protoplast ^e		21.3 ± 6.2				14.5 ± 5.1	

^aColumn of 3ml volume.

^bColumn of 1ml volume.

^cAverage number of local lesions ± standard error per 10⁶ protoplasts on one half-leaf of *Nicotiana glutinosa*, normalized to a standard TMV solution (0.4ug/ml) which yielded about 60-70 lesions per half-leaf.

^dControl preparation from medium in which uninoculated protoplasts were suspended.

^eControl protoplasts without addition of IVR or control preparation.

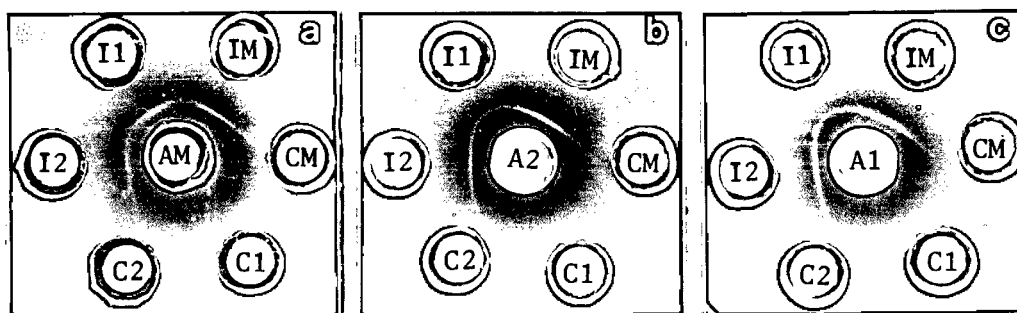


Fig. 4. Agar-gel double diffusion serology showing cross-reactivity between IVR-1 (I-1), IVR-2 (I-2) and mixed IVR (IM). a, antiserum to mixed IVR (AM). b, antiserum to IVR-1 (A1). c, antiserum to IVR-2 (A2). CM-ZnAc₂ preparations from medium of mock-inoculated protoplasts; C1 and C2 parallel control preparation to IVR-1 and IVR-2.

3. An IVR-like compound associated with "green islands" on tobacco infected with cucumber mosaic virus, and obtained directly from plants (in Israel).

INTRODUCTION

Mosaic symptoms are often associated with an uneven distribution of virus, where dark green areas generally contain less infectious virus than light green or yellow areas (1,4,17,18,19). Especially in tobacco leaves infected with cucumber mosaic virus (CMV), Price No. 6, these "green islands" are very distinct (Fig. 5) and were found to contain less than 5% of the infectivity extracted from the yellow areas (12). In older infected plants layers of leaves that have typical yellow mosaic symptoms alternate with leaves that remain virtually symptomless (8,10). Green areas did not contain infectious RNA and only limited amounts of viral antigen could be detected serologically and they were resistant to reinfection with three different strains of CMV but not to infection with tobacco mosaic virus (TMV) (12). These results indicate that resistance of the "green islands" is not due to capture of the superinfecting genome by coat protein as proposed for "cross protection" (3), or to the presence of a strain different from that in yellow areas.

We have reported that protoplasts of a tobacco cultivar in which the infection in the intact plant is localized (resistant), release after inoculation with TMV, a substance(s) that inhibits virus replication (IVR) (13). IVR was not released from non-infected protoplasts of the resistant cultivar, nor from TMV-infected protoplasts of a systemic responding tobacco cultivar (susceptible). IVR was partially purified using ZnAc_2 precipitation, and yielded two biologically active principles, IVR-1 (26,000 MW) and IVR-2 (57,000 MW), as determined by gel filtration. IVR also inhibited replication of several viruses

when applied to leaf disks of different host plants, indicating that it is neither host - nor virus-specific (5).

We were therefore interested to see whether protoplasts from "green island" tissue also release an inhibitor of virus replication, to compare its properties with those of IVR and to see if this inhibitor termed IGI (inhibitor from "green islands"), can be obtained directly from "green island" leaf tissue. A preliminary report has been presented (15).

MATERIALS AND METHODS

Virus and plants

Nicotiana tabacum L. Xanthi-nc and Samsun were grown in 10 cm pots in the greenhouse, with supplementary lighting providing a photoperiod of 14 h. Three feedings with a complete nutrient solution were given at 14 day intervals, starting one week after transplanting. Two weeks after planting, when plants reached a height of approximately 12 cm, they were transferred to a 23-25°C greenhouse chamber, and their lower leaves were inoculated with CMV strain Price No. 6 (CMV-6) (1.5ug/ml) (16). Purified virus (12) diluted in 0.05 M sodium phosphate buffer, pH 7.5, and kept frozen at -70°C, was used. Healthy plants under similar conditions were used as controls.

Infectivity was assayed on primary leaves of 8- to 11-day-old Vigna sinensis Endl. var. 'Black Eye' plants.

Preparation of protoplasts and inhibitory substance.

Protoplasts were obtained from fully expanded leaves with well developed "green islands" 8-10 days after the last feeding. Similar leaves were sampled from the healthy control plants. Leaves were washed with sterile distilled water, and the lower epidermis was peeled off. The mixed enzyme procedure with overnight incubation was employed, using 0.02-0.03% macerozyme R-10 and 0.15-0.3% cellulase R-10 in incubation medium (VIM) containing 13.5% (w/v) mannitol, 0.2 mM KH_2PO_4 , 1 mM KNO_3 , 0.1 mM MgSO_4 , 10 mM CaCl_2 , 1 mM KI and 0.01 uM CuSO_4 (12). Protoplasts

were washed twice with 13.5% mannitol, floated on 23% sucrose and again washed twice with mannitol.

For inoculation of protoplasts purified CMV (2 ug/ml) was incubated at 25°C for 5 min in 0.02 M potassium citrate buffer, pH 5.2, containing poly-L-ornithine (2 ug/ml) (Sigma, approx. MW=120,000) (PL0). After that, 5×10^6 protoplasts in 10 ml 13.5% mannitol were added to 10 ml of the infection medium, and the mixture was incubated at 25°C for 15 min while being rocked gently. Control batches were sham-inoculated with the same infection medium but without CMV. Protoplasts were then washed twice with 13.5% mannitol containing 0.1 mM CaCl_2 followed by a wash with VIM containing carbenicillin (200 ug/ml) and mycostatin (25 units/ml). Protoplasts were suspended in VIM containing the antibiotics at a concentration of 1×10^5 protoplasts/ml and divided into 10-ml portions in 100-ml Erlenmeyer flasks. They were then incubated at 25°C under continuous illumination of about 2000 lx. After 72 h the incubation media were collected and sterilized by filtration through Millipore 0.2-um filters after removal of the protoplasts by centrifugation at 35 g for 6 min. No residual infectivity was detected in the incubation medium from the inoculated protoplasts. The number of infected protoplasts after inoculation of healthy protoplasts with CMV was determined several times using the fluorescent antibody technique and found to be in the 80-90% range. Preparations of IGI obtained from 10^6 protoplasts, incubated for 72 h after inoculation, will be termed as one "unit."

Assays of inhibitory potency.

To test the potency of inhibitory activity in the incubation medium (IM), newly prepared Xanthi-nc and Samsun protoplasts were inoculated with CMV and TMV (13), respectively, and after 5 h or more were suspended in IM or control medium. The protoplasts at a concentration of 10^5 /ml in 10 ml of medium, were incubated for 72 h at 25°C, under 2000 lx. They were then collected by centrifugation and homogenized for 2.5 min in 4 ml of 1% K_2HPO_4

for CMV or in 0.05 M phosphate buffer, pH 7.5, for TMV in the cold. The CMV homogenates were inoculated on 8-10 primary leaves of Vigna sinensis var. Blackeye and compared with a standard solution of partially purified CMV on the opposite primary leaf.

TMV homogenates were each inoculated on 12 half-leaves of Nicotiana glutinosa plants and compared with a standard solution of purified TMV on the opposite half-leaf. TMV lesion counts were adjusted to 10^6 live protoplasts and calibrated using the following equation (14):

$$\begin{aligned} & \text{calibrated number of lesion per half-leaf for homogenate} \\ & \quad \text{mean number of lesions per half-leaf produced by} \\ & \quad \text{homogenate} \\ = & \frac{\text{mean number of lesions produced by standard TMV on opposite}}{\text{half-leaf}} \\ & \times \text{mean number of lesions per half-leaf produced by standard TMV} \\ & \quad \text{from all experiments.} \end{aligned}$$

Calibration of CMV lesion counts were done similarly except that whole opposite leaves were used. The survival rate of the protoplasts after 72 h was generally 90%; those experiments with survival rates lower than 80% were discarded.

Inhibitory potency of the semipurified inhibitor (see below) was also assayed on leaf disks, as described before (5). Leaf disks were obtained from Xanthi-nc plants inoculated 5 h previously with CMV-6 and from Samsun plants inoculated similarly with TMV. After various times of incubation at 25°C, two disks from each IGI and control test were washed with distilled water and homogenized in 1% K_2HPO_4 or 0.01 M phosphate buffer, pH 7.0, for CMV or TMV, respectively; their infectivity was assayed as described above for protoplast homogenates. In some protoplasts

and leaf disks samples, virus titer was also determined by enzyme-linked immunosorbent assay (ELISA) (2), calibrated to purified CMV or TMV.

Partial purification of IGI.

IGI was partially purified from the protoplast incubation medium, using ZnAc_2 precipitation, as described previously for IVR purification (13). Further purification and estimation of the molecular weight of IGI was obtained by gel filtration on Sephadex G-75 (Superfine, Pharmacia) (13,20).

Serological comparison of IGI with IVR.

Antisera from rabbits prepared against IVR-1 (MW 26,000), IVR-2 (MW 57,000) and against a mixture of IVR-1 and IVR-2 ("combined antiserum") (unpublished results) were used in agar-gel diffusion tests. The serological differentiation index (SDI) was determined by indirect enzyme-linked immunosorbent assay (ELISA) according to Jaegle and Van Regenmortel (9), using the combined IVR antiserum. SDI values were determined by comparing the antiserum dilutions that lead to the same absorbance measurements when homologous and heterologous preparations (IVR and IGI) are assayed by ELISA. In these tests IVR and IGI were applied directly to the ELISA plates. After that, attachment sites on the plastic were saturated with 2% bovine serum albumin before adding IVR-antiserum. Subsequently, goat anti-rabbit conjugate (Miles-Yeda) was used.

RESULTS

Release of inhibitor(s) from "green island" protoplasts (IGI) and partial purification

Table 5 summarizes the data on IGI released into the medium from the protoplasts obtained from "green island" tissue, with and without superinoculation by CMV-6, compared with control media obtained from CMV-6 infected and non-infected Xanthi-nc protoplasts. Crude IGI significantly inhibited virus replication in CMV-infected Xanthi-nc and in TMV-infected Samsun protoplasts, by about 54% and 60%, respectively. Superinoculation of "green island" protoplasts with CMV increased IGI activity somewhat, resulting in inhibition rates of 68% and 62% in CMV-6 and TMV-infected test protoplasts, respectively. This increase in inhibitory activity was not found to be statistically significant, when compared to IGI from "green island" protoplasts.

IGI was partially purified using ZnAc_2 precipitation. These partially purified preparations inhibited CMV and TMV replication in protoplasts by 55% and 58%, respectively. ZnAc_2 preparations from incubation media of superinoculated "green island" protoplasts gave higher inhibition rates, 66%, for the two viruses. This increase was statistically significant when compared to IGI from "green-islands" protoplasts.

ZnAc_2 preparations were also assayed on leaf tissue disks from Xanthi-nc inoculated with CMV and from Samsun inoculated with TMV. Results are summarized in Table 6. ZnAc_2 preparations

inhibited CMV in leaf disks by 79% and 57% when assayed after 48 and 72 h, respectively. Preparations from superinoculated "green island" protoplasts resulted in a significant increase in inhibition rates reaching more than 85%. For TMV, inhibition rates of 47% and 59% were observed when leaf tissue disks were treated with ZnAc_2 preparation from "green island" protoplast media or from media of superinoculated "green island" protoplasts, respectively. This increase due to superinoculation was statistically significant.

Inhibition rates by IGI, as determined by local lesion assays or by ELISA, were dose-responsive. Results, when 0.5-10 units of ZnAc_2 preparations from incubation media of "green island" protoplasts were assayed both on protoplasts and on leaf tissue disks inoculated with CMV, are summarized in Fig. 6. For comparison, ZnAc_2 preparations (same amount of units) from incubation media of non-inoculated protoplasts were used.

Further purification and molecular weight estimation of IGI

Further purification and an estimation of the molecular weight were obtained by gel filtration on Sephadex G-75. A 38 x 1.5 cm column calibrated with several proteins (13) was used. A lyophilized ZnAc_2 preparation obtained from 3×10^7 superinoculated "green islands" protoplasts (30 units) was dissolved in 1 ml of 0.1 M phosphate buffer, pH 6.0, ($\text{OD}_{280} = 2$), placed on the column, and eluted with the same buffer at a flow rate of 100 ml/h. Fractions of 1 ml each were

collected. A control preparation obtained from a similar number of healthy protoplasts, with the OD_{280} adjusted to 2, was passed through the same column and collected in a similar way. Three successive 1 ml fractions were combined and lyophilized. Before testing for inhibitory potency the samples were dissolved in 0.01 M phosphate buffer, pH 6.0, and dialyzed overnight against VLM. Inhibitory potency was tested on CMV-inoculated protoplasts, in comparison with the respective control.

Inhibitory activity was eluted only at two positions, equivalent to molecular weights of approx. 26,000 (IGI-1) and 57,000 (IGI-2) (Fig. 7). Similar results were obtained when these fractions were assayed on Xanthi-nc and Samsun leaf disks inoculated with CMV and TMV, respectively.

Inactivation of IGI by enzymes

Incubation of IGI-containing solutions, after $ZnAc_2$ precipitation, with trypsin or chymotrypsin markedly reduced its activity. When assayed on CMV-infected Xanthi-nc leaf tissue disks IGI reduced virus multiplication measured 72 h after inoculation by 78%, while trypsin- and chymotrypsin-treated IGI gave 16% and 19% inhibition, respectively, compared with the control preparation treated similarly with the enzymes. No significant differences in virus titers were observed between disks treated with control preparations incubated with the enzyme and those incubated without the enzymes. Trypsin and

chymotrypsin inactivated by heating the enzyme for 15 min at 100°C reduced IGI activity only slightly.

Recovery of IGI from "green island" leaf tissue

IGI could be obtained directly from "green island" leaf tissue, as follows: 20 g of "green island" tissue was homogenated in 0.1 M borate buffer, pH 9, containing 0.1% thioglycolic acid, at a ratio of 1:2 (w/v), with the aid of an Omni-Mixer. The slurry was passed through gauze and centrifuged for 20 min at 3500 g. The supernatant was first dialyzed overnight against 0.1 M glycine buffer, pH 2.5, and then for 24 h against 0.01 M phosphate buffer, pH 7. The solution was then centrifuged for 20 min at 3500 g, the precipitate discarded, and ZnAc_2 was added to the supernatant as described previously (13).

The precipitate was dissolved in 1 ml of 0.01 M phosphate buffer, pH 7, and dialyzed overnight against VIM (without mannitol). The partially purified preparations were assayed on CMV- and TMV-infected protoplasts as usual, in comparison with control preparations obtained similarly from healthy leaf tissue. With 40 μl , roughly corresponding to 3 units of IGI (based on calculation of the number of cells per g leaf tissue), inhibition rates of 69% and 57% (averages of three experiments) were obtained when assayed on CMV- and TMV-infected protoplasts, respectively, 72 h after inoculation. Such preparations were also assayed on CMV-infected Xanthi-nc and TMV-infected Samsun leaf tissue disks. Inhibition rates (averages of three or four

experiments) for CMV were 81% and 59%, when disks were assayed after 48 and 72 h, respectively; the rates for TMV were 75 and 72%, respectively.

When these ZnAc_2 preparations, obtained directly from "green island" leaf tissue, were passed through a Sephadex G-75 column, peaks of biological activity were eluted at two positions, equivalent to molecular weights of approx. 26,000 and 57,000.

IGI from leaves was also sensitive to trypsin and chymotrypsin. When assayed on CMV-infected leaf disks, IGI reduced virus multiplication (measured by local lesion assays) 72 h after inoculation by 70%; while trypsin- and chymotrypsin-treated IGI gave 11% and 29% inhibition, respectively, compared with the control preparation treated similarly with the enzymes. No differences in virus titers were observed between leaf disks treated with control preparations incubated with the enzymes and those incubated without the enzymes. Trypsin and chymotrypsin inactivated by heating the enzyme for 15 min at 100°C did not reduce IGI activity, which remained at 71% and 82%, respectively.

IGI was also obtained from "green island" leaf tissue of Samsun tobacco plants inoculated with CMV-6. Development of symptoms and "green island" on Samsun was similar to that on Xanthi-nc. Extraction of IGI and partial purification from Samsun leaf tissue were as described above for that from Xanthi-nc. Partially purified preparations after ZnAc_2 inhibited CMV replication by 51% when assayed on Xanthi-nc. protoplasts, 72 h

after their inoculation with CMV. When these preparation were assayed on Samsun leaf disks infected with TMV for 72 h, inhibition rates of 56% were observed. These preparations also gave two peaks of biological activity, when eluted from a Sephadex G-75 column, at positions equivalent to molecular weights of approx. 27,000 and 56,000.

Susceptibility of "green island" protoplasts to infection by TMV and CMV

Protoplasts obtained from "green island" leaf tissue were susceptible to infection by TMV but not by CMV. When such protoplasts were inoculated with TMV, the average number of local lesions obtained after 24, 48 and 72 h were 10.6, 21.9 and 39.1, respectively, compared with 11.2, 23.2 and 38.5 when healthy protoplasts were inoculated with TMV and assayed at the respective times. (Data are the average number of local lesions on one half-leaf on N. glutinosa from four experiments, normalized as described in footnote b to Table 5.)

Inoculation of such protoplasts with CMV, using two strains, CMV-6 and CMV-T (6), did not result in an increase in virus titer when assayed after 72 h. Inoculation of healthy protoplasts with either one of these strains resulted in infectivity titers after 72 h of about 20 lesions per primary leaf of V. sinensis (see footnote b to Table 5).

Serological comparison of IGI with IVR

IGI-1 (MW 26,000), IGI-2 (MW 57,000) and a mixture of IGI-1 and IGI-2, all from Xanthi-nc, "green island" leaf tissue were compared in agar gel diffusion tests with IVR-1, IVR-2 and the mixture of IVR-1 and IVR-2, obtained from Samsun NN, using antisera against IVR-1, IVR-2 and the "combined antiserum". Five units dissolved in 0.2 ml 0.1 M phosphate buffer, pH 7, were applied to each well. Clear precipitation lines were observed between IGI-1 and the antisera to IVR-1 and IVR-2 (Fig 8 a,b). These lines fused completely, without spurs, with the precipitation lines obtained between IVR-1 and IVR-2 and the respective antisera. Similarly, clear precipitation lines were obtained between IGI-2 and the antisera against IVR-1 and IVR-2 (Fig 8 c,d). These lines also were confluent with the lines obtained between IVR-1 and IVR-2. However, an additional faint line was observed between IGI-2 and the two antisera (Fig. 8 c,d). The mixture of IGI-1 and IGI-2 was also compared to the mixture of IVR-1 and IVR-2, using the "combined antiserum". The major precipitation line fused with that obtained between IVR and the IVR antiserum, but additional lines between IGI and the IVR antiserum were observed (Fig. 8e). No precipitation lines were observed between IGI-1, IGI-2 and their mixture, obtained from Samsun "green island" leaf tissue, and the respective IVR-antisera, even when up to 20 units per well were applied.

For determination of SDI, one unit of IGI from Xanthi-nc or IVR per well was used (30 units of IVR and IGI gave OD₂₈₀ values

of 0.13 and 0.14, respectively). Results, averages of four experiments, with two wells for each point, are summarized in Fig. 9. The SDI at OD 1.0 was 1.8.

These results indicate that serologically, IGI from Xanthi-nc highly resembles IVR, and that the two biologically active fractions IGI-1 and IGI-2, similar to IVR-1 and IVR-2 (unpublished) are serologically very close. IGI from Samsun "green islands" was serologically different.

DISCUSSION

In our previous work it was shown that resistance responsible for localizing the infection of TMV in a tobacco cultivar (local lesions), is associated with an inhibitor of virus replication - IVR (5,7,13). IVR inhibited virus replication in protoplasts, leaf tissue disks and intact leaves. In the present work we found that a substance inhibiting virus replication, IGI, is associated with another resistance phenomenon - the "green islands" - developing in tobacco plants inoculated with CMV-6. "Green island" tissue contains only minute amounts of CMV but is highly resistant to reinfection with three strains of CMV (12). Protoplasts obtained from "green island" tissue were found to release a substance(s) (IGI) that inhibits CMV and TMV replication in both protoplasts and leaf tissue disks. IGI, similarly to IVR, affects virus replication, as IGI was applied at least 5 h after inoculation of the test protoplasts and disks. This test procedure eliminates the possibility that the effect is

due to inhibition of infection, which could result when the substance to be tested is either applied before inoculation or mixed with the virus (11).

IGI resembles IVR in many of its properties: IGI was not virus-specific, inhibiting CMV and TMV; it was partially purified by ZnAc_2 precipitation; was sensitive to trypsin and chymotrypsin, with two biologically active principles of molecular weights of approx. 26,000 and 57,000; and its effect on virus replication in both protoplasts and leaf tissue disks was dose-responsive. IGI was also obtained directly from "green island" leaf tissue, which eliminates the necessity of the laborious procedure of obtaining IGI via protoplast incubation.

IGI from Xanthi-nc "green-islands" highly resembles IVR in its antigenic properties. The precipitation lines obtained between IGI-1 and IGI-2 and the respective antisera, fused with those obtained between IVR-1 or IVR-2 and the two antisera, without any spur formation though, an additional faint line was present between IGI-2 and the two antisera, not observed in the respective controls, or between IVR-2 and the antiserum to IVR-2 (Fig. 8 c,d). Also, the precipitation lines between IGI-1 with the antisera and IGI-2 and the antisera fused completely. This indicates the presence of identical serological determinants in IGI-1 to IVR-1, and in IGI-1 to IVR-2, as well as between IGI-1 and IGI-2. Therefore, as suggested for IVR (3), IGI-2 seems to be a dimer of the IGI-1 fraction.

When the "combined antiserum" was evaluated against the mixture of IGI-1 and IGI-2 in comparison to a mixture of IVR-1 and IVR-2 additional precipitation lines were observed between IGI and the IVR antiserum, not present between IVR and the IVR antiserum, or in the respective controls. This, as well as the additional faint precipitation line observed between IGI-2 and two antisera and the difference in the SDI values, may indicate a certain non-identity between IGI and IVR, though the dissimilarity in the SDI values may be due to some difference in the concentrations of the antigens.

IGI from Samsun "green islands" differed serologically from IVR and IGI from Xanthi-nc, though the biological activity was also associated with two fractions of 26,000 and 57,000 MW.

Protoplasts from "green islands" were resistant to infection with CMV, similar to "green island" leaf tissue, which was previously found to be resistant to reinfection with three strains of CMV (12). These "green island" protoplasts (which release IGI) were susceptible to infection by TMV, similar to the previously observed susceptibility of "green island" leaf tissue (which contains IGI) to infection by TMV (12). The finding that IGI inhibits TMV multiplication when applied after inoculation of healthy protoplasts or leaf tissue disks, but not in "green island" protoplasts or leaf tissue, (based on lesion numbers) (12) cannot be explained at present.

Whether IGI-like compounds produced after activation of host genes are directly responsible for the resistance in "green islands", or whether interference is due to virus-dependent satellite replicating RNAs (as CARNA 5 - CMV associated RNA 5) (10), or by some other mechanism is still an open question.

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FIG. 5. Leaf of Nicotiana tabacum Xanthi-nc infected with CMV-6, with distinct separate green and yellow areas.

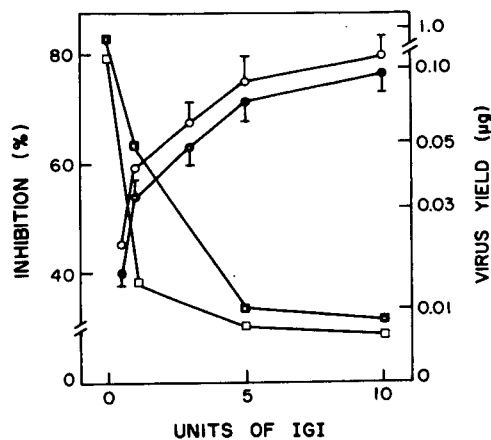


FIG. 6. Effect of IGI dosage on inhibition rates and virus yield (as measured by ELISA) of CMV in protoplasts (o inhibition rate \pm standard deviation; \square virus yield - $\mu\text{g}/10^6$ protoplasts) and leaf disks (o inhibition rate \pm standard deviation; \square virus yield $\mu\text{g}/\text{disk}$) of N. tabaccum Xanthi-nc.

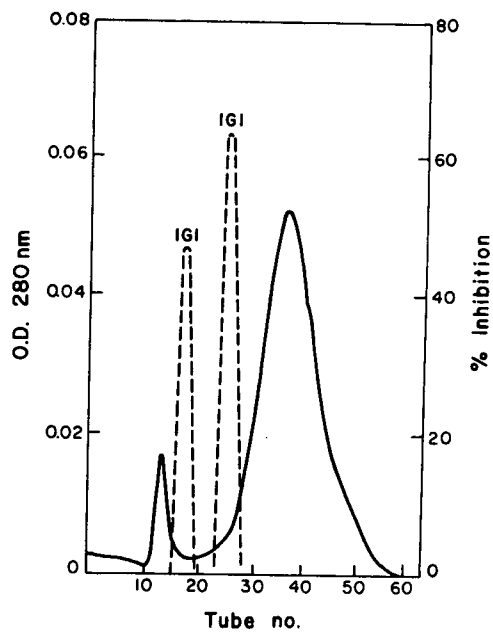


FIG. 7. Column chromatographic separation of IGI on Sephadex G-75. (—) OD_{280} ; (- - - - -) inhibition of CMV replication in protoplasts infected with CMV.

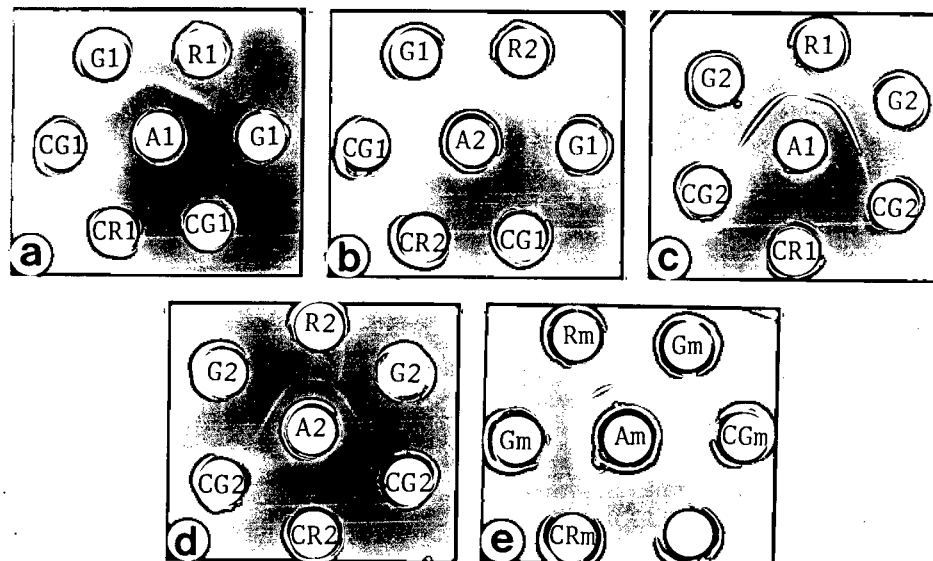


FIG. 8. Agar gel double diffusion serology between IGI, IVR and antiserum to IVR. IGI-1 (G1), IGI-2 (G2), mixture of IGI-1 and IGI-2 (Gm), IVR-1 (R1), IVR-2 (R2), mixture of IVR-1 and IVR-2 (Rm). Antiserum against IVR-1 (A1), IVR-2 (A2) and against mixture of IVR-1 and IVR-2 (combined antiserum) (Am). CG1, CG2, CGm, CR1, CR2, CRm - parallel control preparation to IGI and IVR from mock inoculated protoplasts.

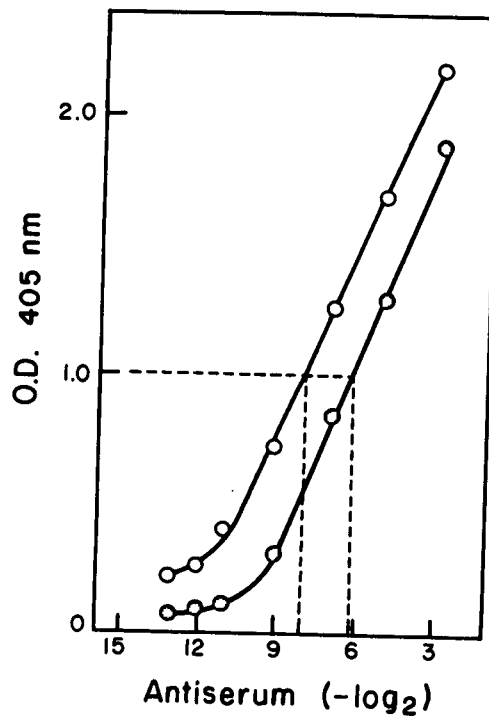


FIG. 9. Determination of serological differentiation index (SDI) values between IGI (-o-) and IVR (-o-) by indirect ELISA, using an antiserum to IVR. The SDI value was calculated from antiserum dilutions corresponding to $OD_{405}=1.0$. The SDI value was $8.0 - 6.2 = 1.8$.

TABLE 5

EFFECT OF INHIBITORY SUBSTANCES (IGI) IN INCUBATION MEDIUM FROM XANTHI-NC "GREEN ISLAND"
PROTOPLASTS - CRUDE AND PARTIALLY PURIFIED BY ZnAc₂ - ON REPLICATION OF CMV AND TMV
IN PROTOPLASTS^a

Test Virus and System	Infectivity ^b from protoplasts suspended in incubation medium from				Control protoplasts ^c
	"Green Island" protoplasts inoculated with CMV	"Green Island" protoplasts inoculated with CMV	Healthy protoplasts inoculated with CMV	Healthy control protoplasts non-inoculated	
CMV-Xanthi-nc protoplast-crude incubation medium	4.8±0.49	3.4±1.03	9.2±1.14	10.5±1.71	15.3±4.74
TMV - Samsun protoplast-crude incubation medium	18.1±4.78	17.1±3.78	33.4±7.42	45 ±9.38	50.2±8.27
CMV-Xanthi-nc protoplast-ZnAc ₂ preparation	6.8±0.51	5.1±0.41	19.1±0.6	15 ±0.5	19.4±1.6
TMV-Samsun protoplast-ZnAc ₂ preparation	20.3±0.80	16.3±0.8	54 ±7.8	48 ±5.0	58 ±6.5

^a Average of two to four experiments.

^b Average number of local lesions and standard error per 10⁶ protoplasts on one primary leaf of *Vigna sinensis* (CMV) or on one half-leaf of *N. glutinosa* (TMV), normalized to a standard CMV (1.5 ug/ml) or TMV (0.2 ug/ml) solution, which yielded about 40 or 65 lesions, respectively

^c Protoplasts without change of incubation medium.

TABLE 6

EFFECT OF IGI ZnAc₂ PREPARATIONS ON REPLICATION OF CMV AND TMV WHEN APPLIED TO
LEAF TISSUE DISKS^a

Infectivity ^b in leaf disks floated on ZnAc ₂ preparation from incubation media from					
Disks assayed (h after inoculation)	"Green Island" "Green Island"		Healthy protoplasts non-inoculated disks ^c		Healthy control Control
	protoplasts inoculated with CMV	protoplasts inoculated with CMV	protoplasts inoculated with CMV	protoplasts inoculated with CMV	
CMV in Xanthi-nc					
48	8.8±3.3	4.4±0.8	30.7±5.25	42.1±6.32	35.7±3.0
72 ^d	7.8±3.1	2.7±1.5	17.9±4.28	18.3±4.7	21.1±3.4
TMV - Samsun					
72 ^e	22.4±0.9	17.4±2.4	41.9±2.2	42 ±4.5	51.2±6.4

- ^a Average of two experiments when leaf tissue disks 5h after inoculation were floated on ZnAc₂ preparations obtained from the incubation medium of 10⁶ protoplasts in 5 ml incubation medium (5).
- ^b Average number of local lesions and standard error on one primary leaf of Vigna sinensis for CMV and on one half-leaf of N. glutinosa for TMV, normalized to a standard CMV (1.5 ug/ml) or TMV (0.2 ug/ml) solution, which yielded about 40 or 65 lesions, respectively.
- ^c Disks floated on incubation medium.
- ^d Homogenate was diluted 1:10.
- ^e Homogenate was diluted 1:50.

4. Obtaining IVR directly from TMV inoculated plants

a. From tobacco (in Israel).

An attempt was made to isolate a compound(s) which inhibits virus replication, similar or identical to IVR.

The compound was prepared as follows:

Samsun NN tobacco leaves were collected 7 days after inoculation with tobacco mosaic virus (TMV). Freshly collected leaves were cut into pieces of 4 to 5 cm². Pieces were infiltrated in vacuo for three to four periods of 30-50 seconds each, with a large excess of cold (4°C) 0,05M phosphate buffer pH 7, 0,1% Mercaptoethanol (Parent, J.G. and A. Asselin, Can. J. Bot. 62: 564-569. 1984). Pieces were gently blotted, rolled up and placed in centrifuge tubes containing an inner matching part with a pierced bottom. This allowed separating the leaf tissue from the intercellular fluid which was collected after centrifugation for 10min at 2000g. The fluid was precipitated with ZnAc₂ (0.02M, final concentration) and processed in a similar manner to the preparation of IVR.

The inhibitory effect of this compound on protoplasts was determined in comparison to a control compound prepared from mock inoculated leaves. An inhibition of 40-70% on virus replication in the protoplast assay was found with this compound. (Table 7).

Partial purification of this compound was done on a Sephadex G-75 column. Two peaks of activity were eluted at positions equivalent to molecular weights of 26,000 and 57,000.

This parallels the peaks of activity found for IVR. These peaks reacted serologically in agar gel diffusion with the antisera prepared against IVR-1 and IVR-2 (Fig 10).

A similar IVR-like compound was obtained from "induced resistant" Samsun NN leaf tissue. In this case, induced resistance was obtained by inoculating both halves of each leaf parallel to the midvein. The IVR-like compound was obtained from the resistant tissue between the inoculated stripes. The inhibitory effect of this compound was similar to the IVR-like compound (Table 7).

TABLE 7

Effect of an IVR-like compound from TMV-infected tobacco NN tissue on TMV replication in Samsun NN protoplasts.

<u>Amount added</u> <u>(units)^a</u>	<u>Infectivity^b</u>		<u>Percentage</u>
	<u>Ivr-like</u>	<u>Control</u>	<u>inhibition</u>
0.2	39	47	17
1	28	51	45
2	22	67	67

^aOne unit represents amount of IVR-like compound obtained for 1g tissue.

^bAverage number of local lesions per 10^6 protoplasts harvested 72h after inoculation and inoculated on *N. glutinosa*. Numbers are normalized to a standard TMV solution (0.1 μ g/ml) which yielded about 50 lesions per half leaf.

TABLE 8

Effect of an IVR-like compound from induced resistant tobacco NN leaf tissue on TMV replication in Samsun NN protoplasts^a.

<u>Compound extracted</u> <u>from</u>	<u>Infectivity</u> ^b	<u>Percentage</u> <u>inhibition</u>
Stripes	1.7	79
between stripes	2.4	70
Control (sham inoculation)	7.9	
Control ^c (protoplasts)	9.6	

^aOne unit (IVR-like compound obtained from lg tissue) added to 10^6 protoplasts 4 hrs after inoculation.

^bAverage number of local lesions per 10^6 protoplasts harvested 72hr after inoculation and inoculated on N. glutinosa. Numbers are normalized to a standard TMV solution (0.1ug/ml) which yielded about 50 lesions per half leaf.

^cInternal control - TMV inoculated protoplasts with no additions.

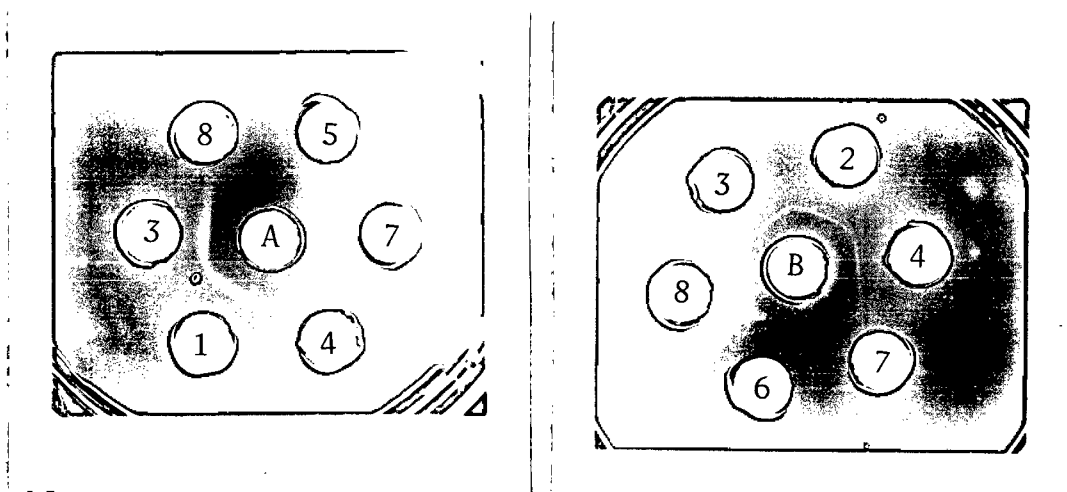


Fig 10. Agar gel double diffusion serology of the IVR-like compound from leaves with antiserum against IVR-I (A) and IVR-II (B).

1. IVR-I
2. IVR-II
3. 57,000 daltons peak of activity - from leaves
4. 26,000 daltons peak of activity - from leaves
- 5,6,7,8. Parallel control preparations for 1,2,3 and 4, respectively.

These results indicate that the IVR-like compound extracted from plants is indeed similar to IVR. It is estimated that 1×10^6 protoplasts are obtained from 1g of leaf tissue. Therefore, the amount obtained from 1g leaf tissue was defined as one unit.

Extraction of the compound from intact plants rather than from protoplasts will allow scaling up the production of this compound in a less laborious and time consuming way and therefore is of obvious advantage.

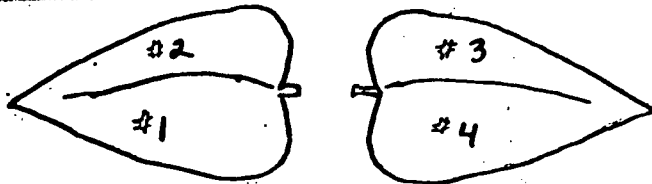
b. Characterization of Intercellular Wash Fluid (IWF) From TMV-Infected Pinto Bean Leaves. (in U.S)

Little information has been published on Pinto Bean intercellular fluid components. Most studies on induced proteins use leaf homogenates from which proteins are extracted.

The goal of this study was to characterize the biological and chemical properties of TMV-infected Pinto Bean intercellular wash fluid.

IWF Extraction Procedure Eleven- to twelve-day old *Phaseolus vulgaris* var. "Pinto" primary leaves were inoculated in the following manner:

Side #1 - TMV + Celite
#2 - No rubbing
#3 - Buffer + Celite
#4 - No rubbing



IWF was extracted at selected intervals after inoculation by vacuum infiltration of cold 0.067M sodium-potassium phosphate buffer, pH 7.0 with 0.1% 2-mercaptoethanol into the half-leaves. Leaves were blotted dry, placed into centrifuge tubes and spun for 8-9 min at 1200g. The IWF was generally stored at -17C prior to use.

IWF Activity Against Virus Establishment IWF, when mixed with TMV and inoculated onto healthy, 11-12-day old Pinto Bean primary leaves, produced variable results in inhibiting virus establishment. At most, a 30% reduction in lesion numbers was observed, with IWF extracts taken 3 and 7 days after inoculation consistently producing that amount of virus inhibition. The low percentage of inhibition did not warrant further experiments testing the relation between the physical characteristics of IWF and virus establishment.

IWF Activity Against Virus Replication Eleven- to twelve-day old Pinto Bean Primary leaves were inoculated with TMV 6-9 hrs prior to being infiltrated with Day-7 IWF extracts. Half-leaves were submerged in a mixture of IWF and buffer and placed under vacuum. Leaves were kept on moist towelling in a 20-23C growth chamber.

Lesion numbers and diameters were measured 4-6 days later. Overall, lesion numbers were decreased 60-69% by the four-treatment extracts compared to the control (Table 1).

Average lesion diameters changed little as compared to the control, although the appearance of the larger lesion size classes was diminished in the treatments using side #1 and side #2 IWF extracts (Table 2).

Table 1. Lesion Size-Class Frequency Percentages Using Day-7 IWF Extracts Infiltrated into Pinto Bean Leaves 6-9 Hrs After TMV Inoculation.

Size Class -1 (x10 mm)	TREATMENT				
	Control	1	2	3	4
1	11.9	16.6	10.9	11.6	5.4
2	26.7	22.1	32.7	22.3	17.3
3	20.0	23.5	32.3	26.5	26.9
4	11.5	14.7	11.7	12.1	16.5
5	10.7	13.4	8.2	13.5	19.2
6	5.2	5.1	2.7	7.4	5.4
7	6.3	3.2	0.8	2.3	5.0
8	3.3	1.4	0.4	2.3	1.5
9	4.4	---	0.4	1.9	2.7

Average of 6 trials.

Total leaves used: Control (N=36), Treatments (N=47-48).

Total lesion diameters measured: Control (N=270),

Treatments (N=215-260).

Table 2. Comparison of Average Lesion Number and Diameters Using Day-7 IWF Extracts Applied 6-9 Hrs After Inoculation with TMV.

	TREATMENT				
	Control	1	2	3	4
=====					
Average Lesion Number	60.0	22.1	23.9	18.4	18.3
Average Lesion Diameter					
(x10 ⁻¹ mm)	3.3	3.2	2.9	3.5	3.9
=====					

IWE Protein Contents Protein contents of IWF extracts made at different intervals after virus inoculation, were measured using the Bradford Protein Method. Peak increases in protein content occurred in the 12-hr, Day-4, Day-7- and Day-10 extracts. Treatment #1 of Day-7 IWF usually contained the greatest amount of total protein (Table 3).

Table 3. Comparison of IWF Protein Contents (mg/ml) Taken at Selected Times After TMV Inoculation.

Extraction Time	Treatment				I	Percent Difference			
	1	2	3	4		1:3	1:4	2:4	3:4
=====									
0 hrs	38.6	32.6	37.7	50.9	I	+2	-24	-36	-25
12 hrs	14.4	15.2	9.2	9.2	I	+57	+51	+60	-3
24 hrs	12.3	----	12.0	10.6	I	+3	+16	---	-13
36 hrs	16.3	13.5	20.8	15.1	I	-22	+8	-11	+38
48 hrs	15.2	17.9	23.0	13.6	I	-34	+12	+32	+69
4 days	57.0	36.7	15.7	10.5	I	+263	+443	+249	+49
5 days	26.9	28.1	28.6	17.5	I	-6	+54	+61	+63
7 days	63.0	34.7	30.0	19.5	I	+110	+223	+78	+54
8 days	35.2	23.5	21.4	22.5	I	+64	+56	+4	-5
9 days	35.8	18.5	17.3	16.1	I	+107	+122	+15	+7
10 days	55.1	19.7	18.0	14.3	I	+206	+285	+38	+26

Preliminary protein content studies of IWF extracts taken at selected days from Pinto Bean plants that were only rubbed with buffer and Celite gave interesting results. A slight periodicity over time still appears in the levels of intercellular proteins after only wounding half of a single primary leaf on a plant. The results represent only one trial using 12-15 plants per extraction day. Treatment letters; i.e., side a,b,c,d, correspond to the same sides of Pinto Bean primary leaves as in the other experiments using sides 1,2,3,4, respectively. Side c was the only side rubbed with buffer (Table 4).

Table 4. IWF Protein Contents (mg/ml) of Buffer-inoculated Pinto Bean Primary Leaves.

Extraction Time	Treatment				Percent Difference		
	a	b	c	d	1	a:c	a:d
=====							
3 days	312	239	314	277	1	-1	+13
4 days	379	408	342	249	1	+11	+52
5 days	363	309	405	366	1	-10	-1
6 days	376	401	206	159	1	+83	+136
7 days	198	289	154	273	1	+29	-27
=====							

Protein Characterization of IWF IWF extracts were run on an SDS-PAGE gel (Judd,1982) and produced 5 bands that showed increased protein levels resulting from TMV infection. These bands probably did not represent de novo proteins as there were light bands visible, at the same molecular weights, in the healthy and control IWF extracts. The apparent molecular weights of the five bands were 36,500, 34,000, 33,000, 28,750 and 26,500 daltons.

These results indicate that general cell leakage is not a direct result of virus infection because there is no overall increase in the density of staining of side #1 treatments compared to the other treatments. It is hypothesized that the plant may be selectively releasing proteins into the intercellular spaces as a result of TMV infection. Isolation of an inhibitor from IWF was not undertaken.

Discussion Proteins which were increased after the application of stress, in the form of viral or fungal infection, chemicals or wounding, have been studied by many researchers. A variety of names have been given to these proteins, depending on the researcher and species of plant used.

PR or "h" proteins have been used to designate induced proteins in *Nicotiana* sp., whereas PS, P and p proteins have been used to designate induced proteins in *Ephaseolus* sp., *Cynura* sp. and *Lycopersicon* sp., respectively.

Van Loon, et al(1983), using serological techniques, have found that although many of these induced proteins share the same range of molecular weights, the proteins are genus-specific. Antisera to some of the tobacco h-proteins did not react serologically to any of the induced proteins tested from other genera. However, that does not preclude the possibility that the proteins which increased in concentration after virus infection in our studies, could be similar, if not in chemical makeup then in function, to the PS proteins or even the h-proteins.

References

125

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- Van Loon, L.C., S. Gianinazzi, R.F. White, Y. Abu-Jawdah, P. Ahl, J.F. Antoniw, T. Boller, A. Camacho-Henriquez, V. Conejero, J.C. Coussirat, R.W. Goodman, E. Maiss, P. Redolfi and T.M.A. Wilson. 1983. Electrophoretic and serological comparisons of pathogenesis-related (b) proteins from different plant species. *Neth.J.Pl.Path.* 89:293-303.

5. Preliminary attempts to purify IVR by high performance liquid chromatography. (in collaboration with Prof. Y. Burstein and Dr. V. Buchner, Weizmann Institute) (in Israel).

Two hundred units of IVR and a parallel control were fractioned on a Sepharex G75 column. The high molecular weight (56.000) and the low molecular weight (26.000) fractions obtained were further purified using high performance liquid chromatography (HPLC).

HPLC was performed on a spectrophysics 8100 liquid chromatography with a column of Aquapore RP-300. The column was first eluted under isocratic conditions using 0.01M sodium perchlorate, 0.1% phosphoric acid and then by a linear gradient of n-propanol in the same buffer (pH 2). Fractions were collected and those showing an obvious peak which was absent or smaller in the healthy control were assayed for their bioactivity in the protoplast system. In the first experiment 3 peaks of the high molecular weight fraction were tested and assayed with the respective controls. The peak eluted from the high molecular weight fraction at 43.53' was found to have 60% inhibition when assayed in the protoplast system (Fig. 11).

In a second experiment the high and low molecular weight fractions were further purified by reversed phase high performance liquid chromatography (RPLC). RPLC was performed on a spectrophysics 8700XR HPLC system, equipped with an Aquapore RP-300 column using a gradient of n-propanol in 0.01M sodium perchlorate 0.1% phosphoric acid. Fractions were collected and assayed as described previously. Two peaks of the high molecular weight fraction with retention times

37.09' and 41.18' were found to have 70 and 50% inhibition respectively and two peaks of the low molecular weight fraction with retention times 41.85' and 43.00' were found to have 62% and 40% inhibition (Fig. 12).

In this experiment, active peaks were eluted at retention times 41.00 and 43.00 both from the high and low molecular weight fractions. This also suggests that the high molecular weight fraction is indeed a dimer of the low molecular weight fraction and is broken to its monomeric form. The elution of active peaks around 42.00 is in agreement with the results obtained in the first experiment.

The active fraction thus obtained was desalted and further purified by rechromatography on the same column, using a gradient of n-propanol in trifluoroacetic acid (TFA).

Several peaks were eluted at retention times 21'28 to 25'82 from the two low molecular weight fractions (Fig. 13). The bioactivity of these peaks as well as a few peaks obtained from the high molecular fraction were evaluated in the protoplast system.

The peaks eluted at 21'28 and 25'82 from the low molecular weight fraction and the peak eluted at 15'86 from the high molecular weight fraction were found to have 60-70% inhibition. These peaks will be further analysed for amino acid composition and sequencing.

Fig. 11. High molecular weight fraction

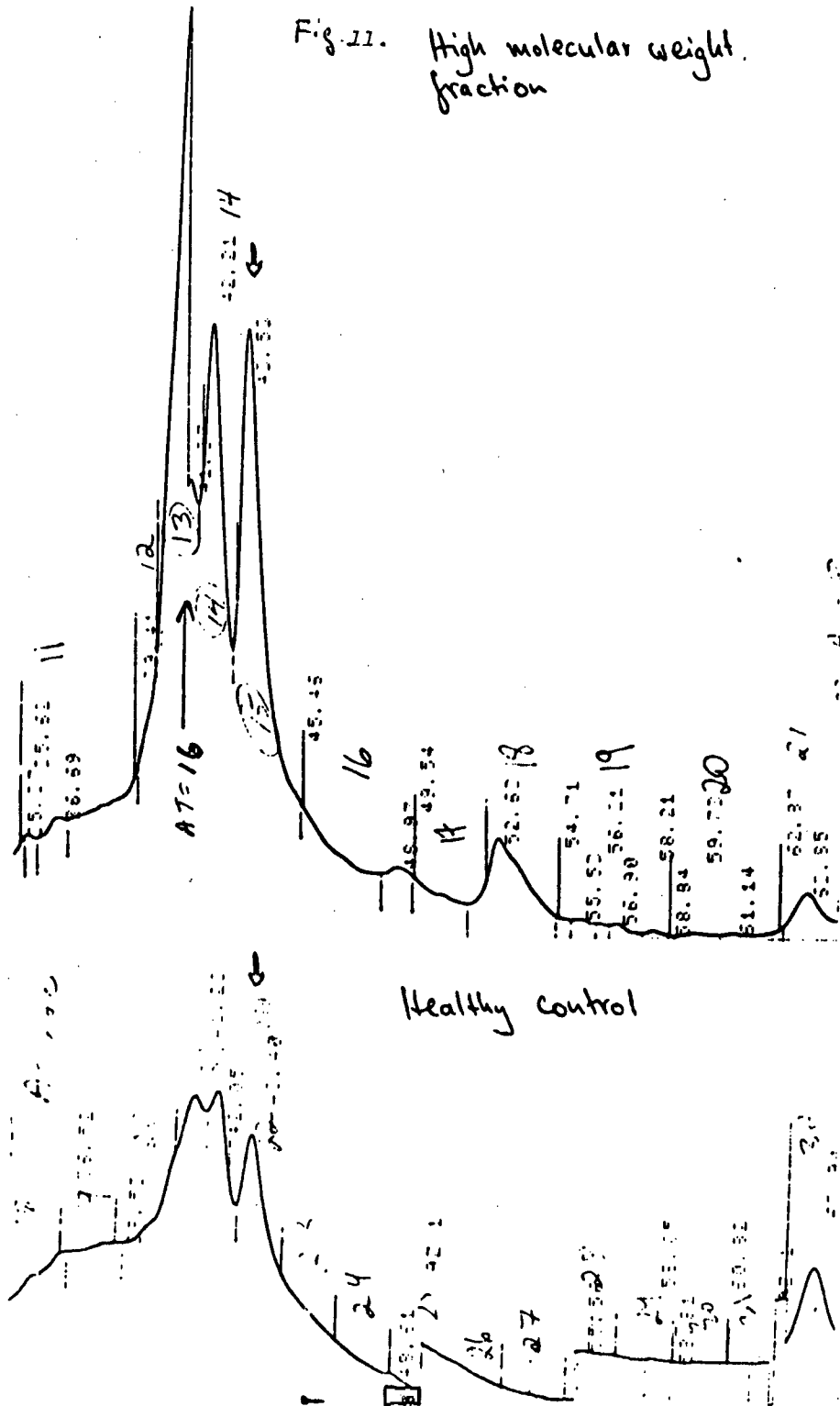
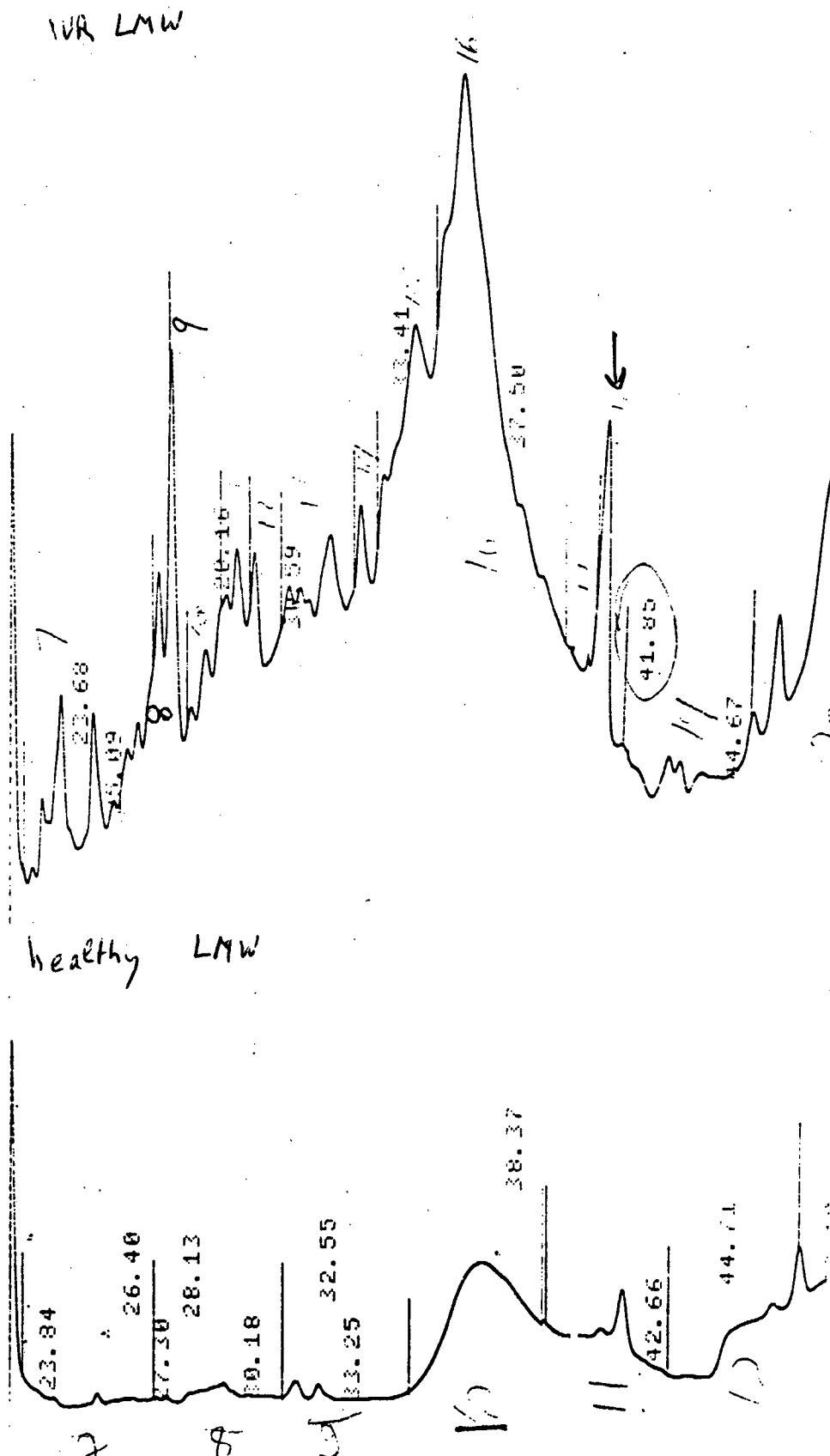
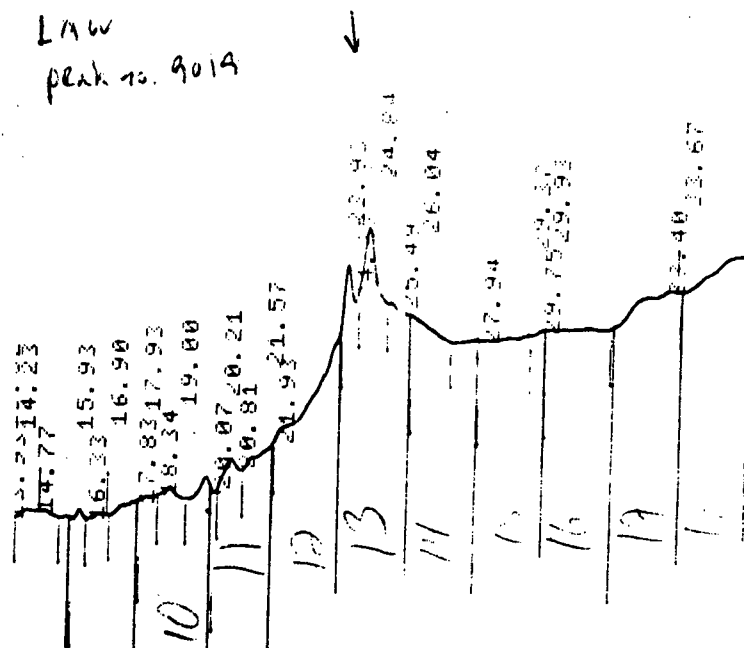
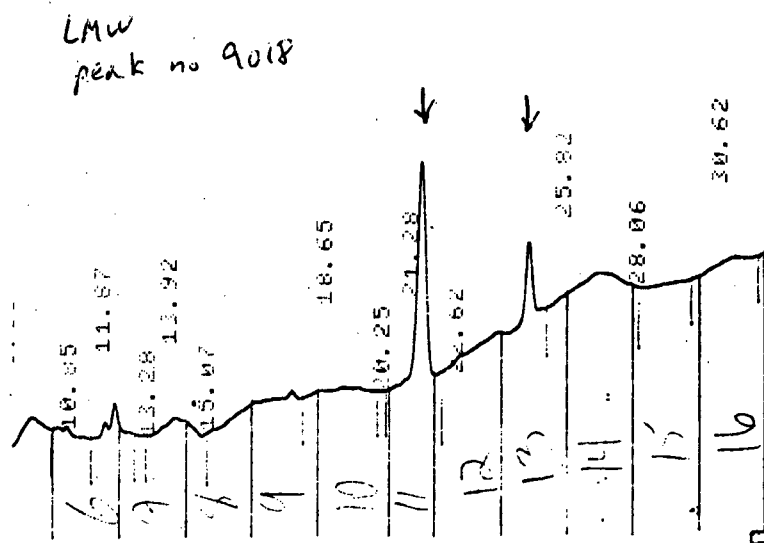


Fig.12 Fractionation of the low molecular weight IVR by reversed phase high performance liquid chromatography (RPLC).



All the fractions were tested for bioactivity.

Fig. 13 Rechromatography of active peaks on a Aquapore RP-300 column following desalting.



Arrows indicate peaks being tested for bioactivity in the protoplast system.

D. ISOLATION OF ANTIVIRAL SUBSTANCES (AVS) (Montana)

General:

Although several methods were tried to isolate AVS from the protoplast culture medium 72 hours after inoculation of the protoplasts with THV, modified Sela's method was found to be most consistent and hence used exclusively.

Protoplasts were isolated from five tobacco species, viz, xnc, ng, snn, xanthi and sam. A mixed enzyme method was used to isolate protoplasts using macerase R-10, cellulase-R-10 and occasionally pectolyase Y-23. Protoplast culture medium (PCM) was collected and dialyzed against distilled water for 24 hours at 4C. To the dialyzed PCM an equal volume of HCP was added and vortexed for 5 minutes. The mixture was then centrifuged at 4000 RPM and the supernatant was subjected to the DEAE-Cellulose column chromatography. In an early experiment the column was eluted with a gradient of PBS (0.1-1M NaCl) and two peaks of antiviral activity were detected at 0.3M NaCl and 0.65M NaCl. In the subsequent experiments the column was washed with 0.1M NaCl, first AVS (AVS-30) was eluted with 5 ml of PBS (0.3M NaCl), the column washed, and then the second AVS (AVS-65) was eluted with 5 ml of PBS (0.65M NaCl). The two fractions were dialyzed against distilled water and were stored frozen at -33C.

DETERMINATION OF ANTIVIRAL ACTIVITY:

1. Using intact plants:

Leaves were inoculated with THV (20ug/ml), 6 hour after inoculation one half of the leaf was injected with AVS and the other half was injected with distilled water. Five to seven days after inoculation lesion number was counted and the lesion area was measured.

Source	Test Plants	Percent Protection			
		Lesion #		Lesion Area	
		AVS-65	AVS-30	AVS-65	AVS-30
XNC	NG	74+-22	46	82+-16	34
	SNN	71	32	88	32
NG	XNC	64	48	92	30
	SNN	68	51	94	30
SNN	NG	78	42	84	33
	XNC	81	40	82	37
SAM	NG	0	27	0	33
	XNC	0	24	0	41
	SNN	--	--	--	--

2. Using protoplasts:

Six hours after inoculation protoplasts were collected and resuspended in the medium containing AVS and incubated for 72 hours. Virus titer was determined by ELISA.

Source	Test Protoplasts	% Protection	
		AVS-65	AVS-30
NG	ANC	90	46
	SNN	92	56

3. PAGE of AVS:

Both native and SDS PAGE were tried, but no band could be detected. A tenfold increase in concentration by lyophilization did not increase PAGE sensitivity.

EFFECTS OF ANTIMETABOLITES:

Antimetabolites were tried in the protoplast system only using NG protoplasts.

1. PI-DMS and AVS:

Ten million protoplasts were inoculated with TMV and suspended in the protoplast culture medium containing either 10ug/ml AMD or 20ug/ml CH. The protoplast were incubated for 72 hours and the AVS was obtained from the protoplast culture medium. AVS was tested in SNN protoplasts for antiviral activity.

AMS Used	Percent Protection	
	AVS-65	AVS-30
AMD	59	34
CH	68	18
Control	89	38

This "0" time application only gave consistent result. AMS applied 1-6 hours PI yielded contradictory data.

2. AVS vs AVS:

Ten million SNN protoplasts were treated with AVS (from ten million NG protoplasts) for 4-6 hours and then the protoplasts were resuspended in the protoplast culture medium and incubated for 72 hours. The protoplast medium was used to isolate AVS. Treatment of the SNN protoplasts with AVS-30 resulted in only 0.3H peak from SNN protoplast medium whereas treatment with AVS-65 resulted in both 0.3H and 0.65H peaks.

EFFECTS OF AVS ON CELL-FREE PROTEIN SYNTHESIS:

Messenger depleted Rabbit Reticulocyte Lysate (RRL) was used as the cell free system and full length TMV-RNA was used as the message. Protein synthesis was determined by measuring the incorporation of ³H-leucine in TCA precipitated protein.

AVS-65 inhibited protein synthesis by 78-83% whereas AVS-30 caused only 7-15% inhibition.

SUMMARY:

The protoplast culture medium seems to contain two types of inhibitors: endogenous and induced. The induced inhibitor inhibits virus replication by 80-94% whereas the endogenous inhibitor inhibits virus replication only by 27-51%.

Cycloheximide and Actinomycin-D partially inhibit the synthesis of the inhibitors indicating the involvement of cellular transcription and translation processes.

Treatment of protoplasts with AVS for 4-6 hours results in the induction of AVS synthesis when the treated protoplasts are incubated in the culture medium further for 72 hours.

The induced and endogenous inhibitors inhibit TMV-RNA directed protein synthesis in RRL system by 78-83% and by 7-15% respectively.

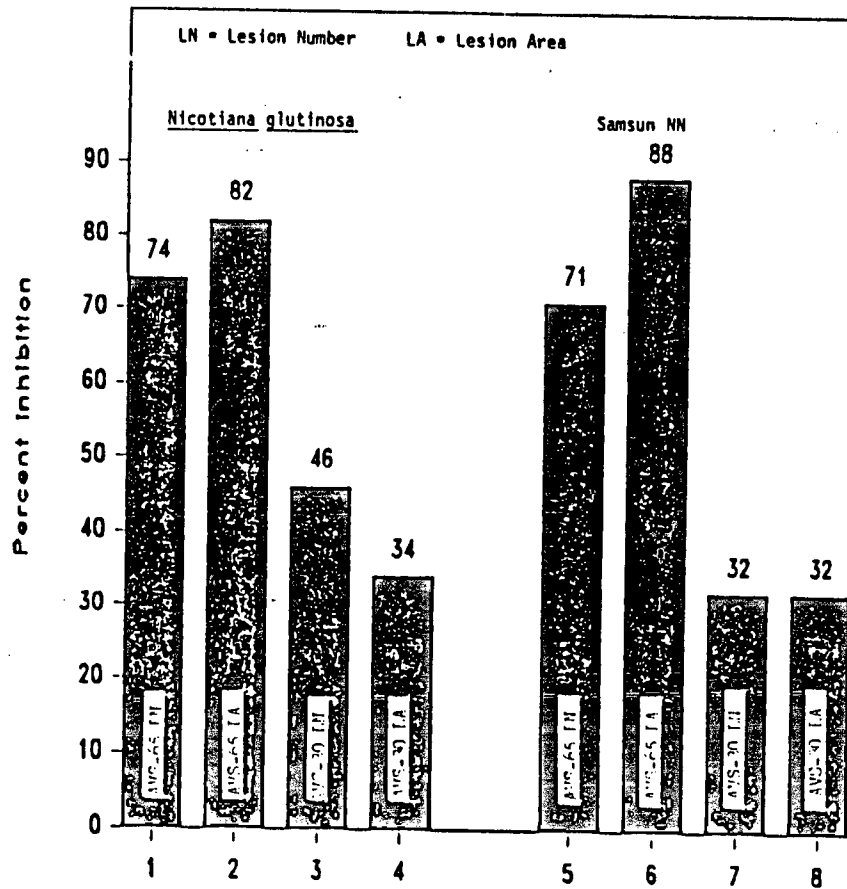


Figure 1. Antiviral activity of AVS isolated from 'Xanthi nc' protoplasts and tested in leaves.

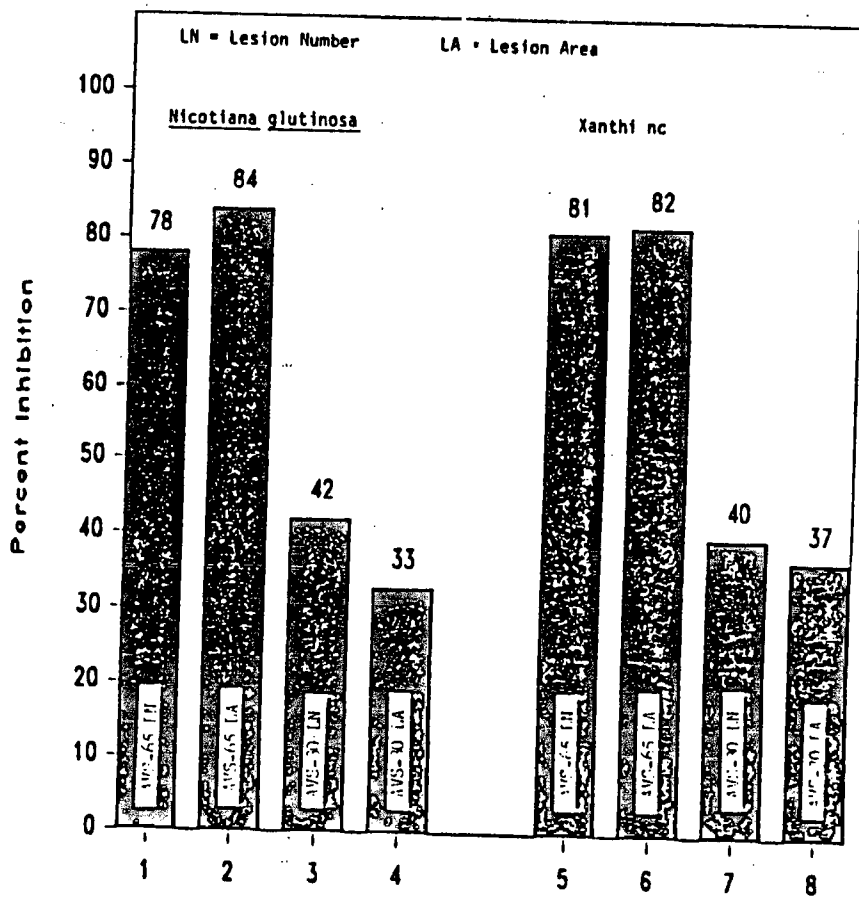


Figure 2. Antiviral activity of AVS isolated from 'Samsun NN' protoplasts and tested in leaves.

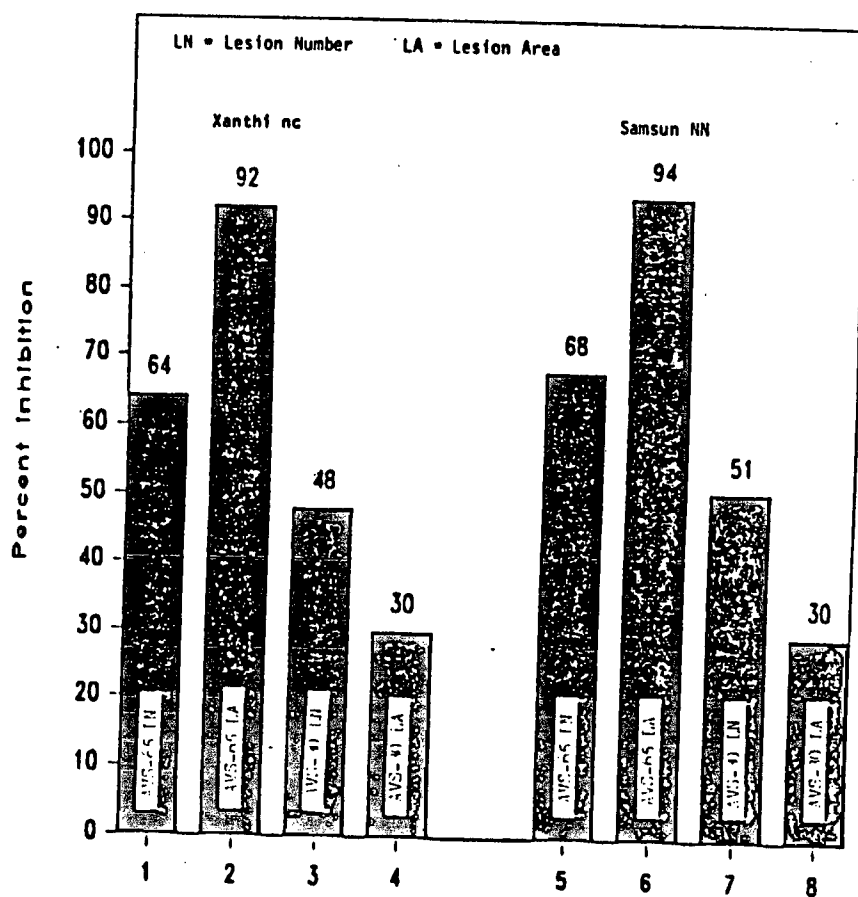


Figure 3. Antiviral activity of AVS isolated from N. glutinosa protoplasts and tested in leaves.

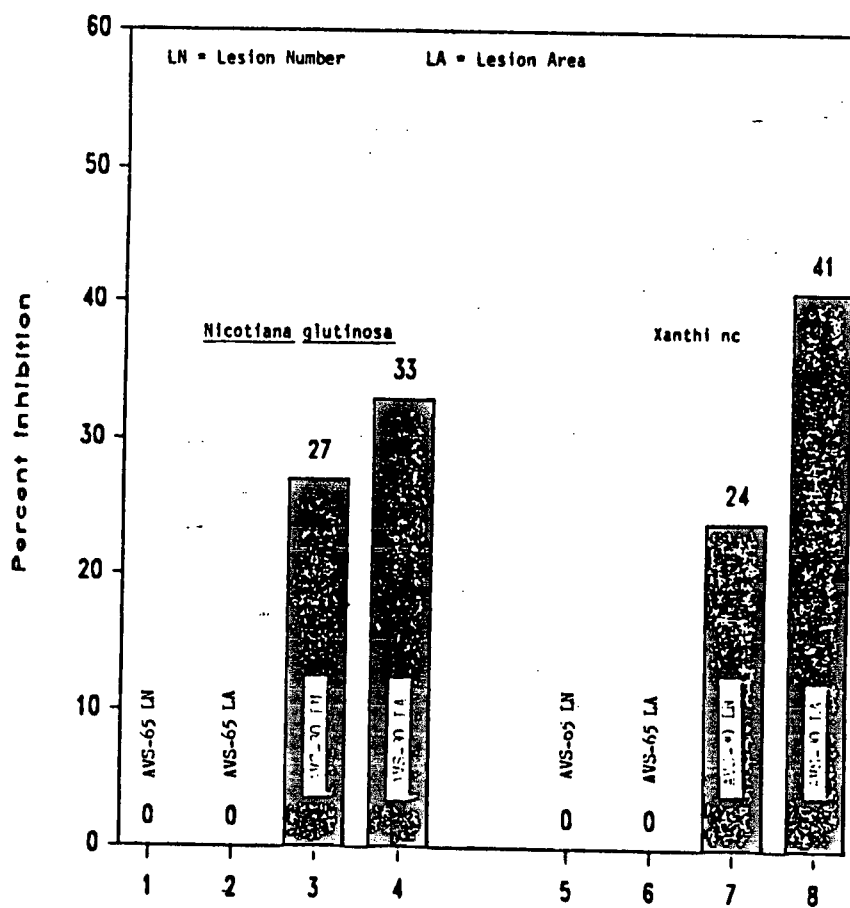


Figure 4. Antiviral activity of AVS isolated from 'Samsun' protoplasts and tested in leaves.

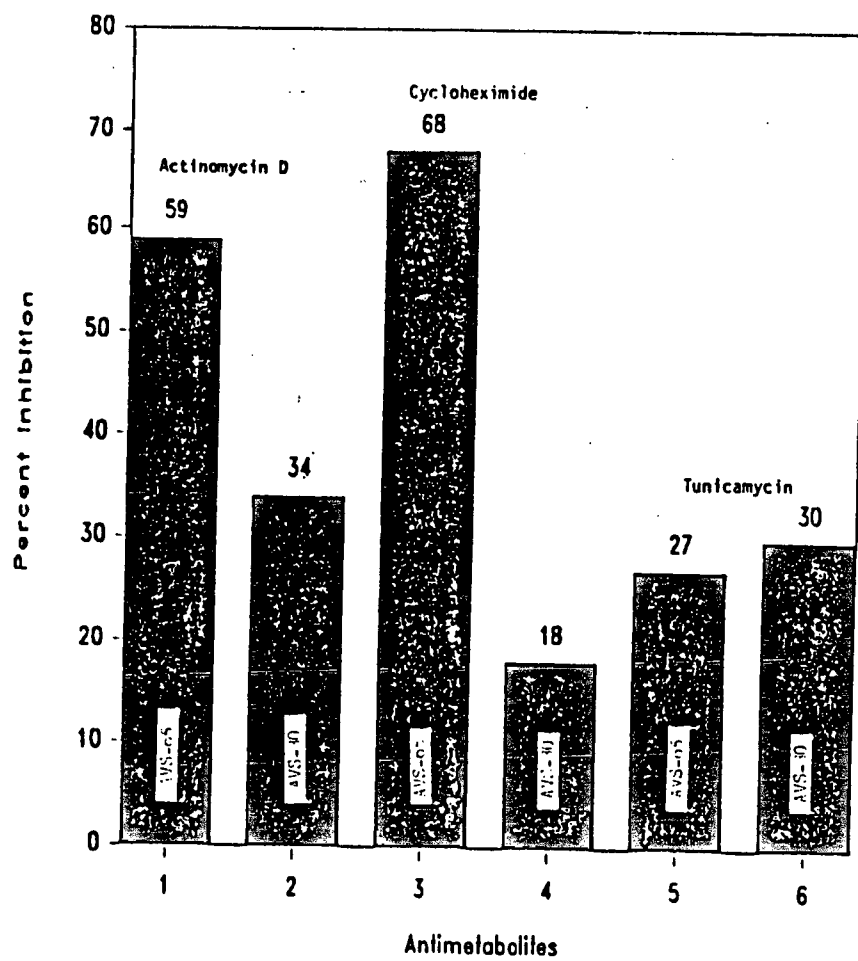


Figure 5 Effects of antimetabolites on AVS activity

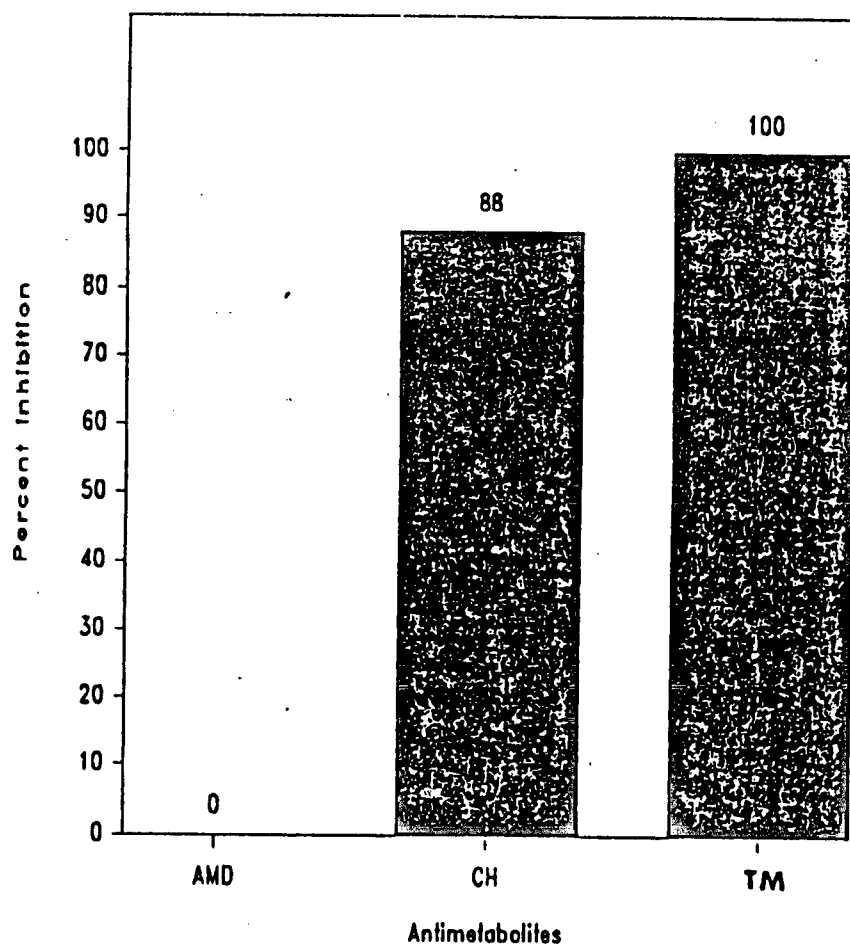


Figure 6. Effects of antimetabolites on TMV-RNA directed *protein* synthesis in the rabbit reticulocyte lysate system.

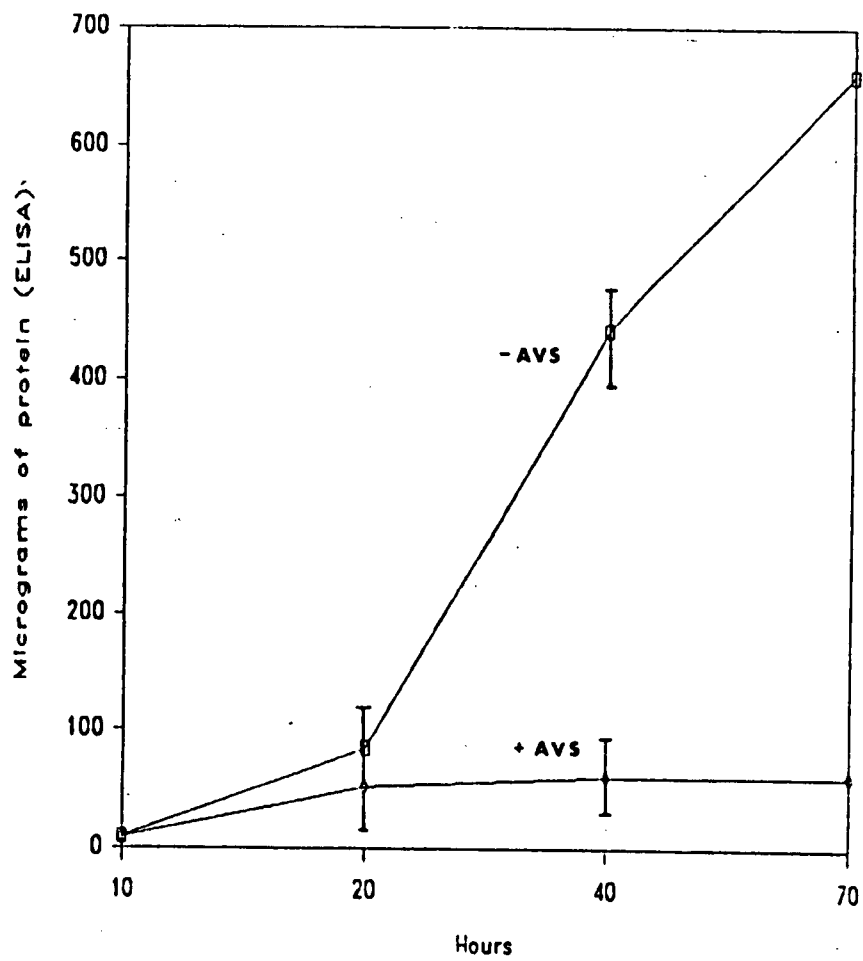


Figure 7. Effect of AVS-65 on the synthesis of TMV coat protein in protoplasts.

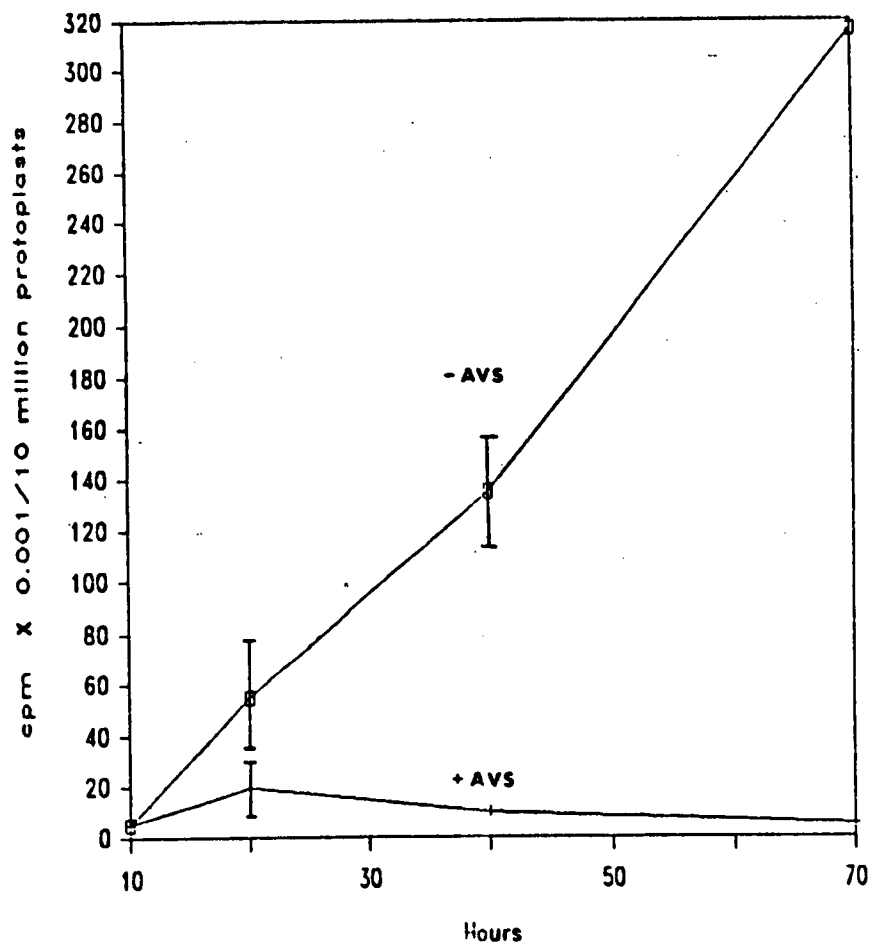


Figure 8. Effect of AVS-65 on the synthesis of TMV genomic-RNA in protoplasts.

ANTIMETABOLITES	PERCENT INHIBITION	
	AVS=65	AVS=30
Actinomycin D (10 ug/ml)	30	3
Cycloheximide (20 ug/ml)	21	20
Tunicamycin (5 ug/ml)	69	8

Table 1. Effectiveness of antimetabolites in inhibiting AVS activity.

ENZYME TREATMENTS	PERCENT INHIBITION
Alkaline phosphatase + AVS-65	31
RNase A + AVS-65	84
RNase T-1 + AVS-65	88
Alpha-glucosidase + AVS-65	41
Beta-glucosidase + AVS-65	88
Trypsin + AVS-65	60
AVS-65 (control)	87

Table 2. Effects of various enzymes on AVS-65.

E. Binding of IVR to Concanavalin A-Sepharose (in Israel).

To see wheather IVR binds to lectin, a column (10x40mm) was packed with Con-A - Sepharose 4B (Pharmacia) and equalibrated with 0.02M Na-phosphate containing 0.15 M NaCl, pH 7.4 (PBS).

IVR (10 units) was dialyzed for 16 hr at 4°C against 100 volumes of PBS and loaded to the column. Washing and elution were performed as follows:

1. Washing with 8 ml of PBS (E₁).
2. Washing with 6 ml of 0.02 M Na-phosphate containing 1M NaCl, pH 7.4 (E₂).
3. Elution with 8 ml of (E₂) containing 0.1 M methyl -D-Manoside (E₃).
4. Elution with 8 ml of (E₃) containing 70% ethylene-glycol (V/V) (E₄).

All the eluates were collected separately.

A control preparation obtained from a similar number of mock-inoculated protoplasts was passed through a similar column, eluted and collected in a similar way.

All fractions were dialyzed three times, against two liters of deionized water. The biological activity of an amount equivalent to

three units was tested on TMV-infected tobacco protoplasts, in comparison with the respective control fraction.

Results of one experiment is given in Table 1. Two peaks of activity were observed after elution with PBS containing 0.1 M manoside (E_3) and E_3 containing ethylene glycol.

Table 1. Lectin chromatography of IVR.

Elution buffer	<u>Infectivity</u>		% Inhibition of infectivity
	IVR	Control medium	
PBS	27	35.9	25%
0.02 Na-phosphate containing 1M NaCl pH 7.4 (E_2)	28.2	37.9	26%
(E_2) containing 0.1 M methyl manoside (E_3)	13.1	31.6	51%
(E_3) containing ethylene glycol	14.9	32.3	54%

These results, which are of a preliminary nature and require further confirmation, may indicate that the IVR protein is glycosylated.

F. Production of IVR Concurrently with Resistance and its Suppression
by Antimetabolites. (in Israel).

Results are summarized in the attached publication.

Virology 127: 475-478, 1983

g. Mode of Action of IVR.

1. Effect of IVR on plasmodesmata number (in Israel).

The mechanism whereby IVR inhibits virus replication is not yet known, but as recent reports implicated changes in plasmodesmata as being associated with virus restriction (Shalla et al., 1982, Leonard & Zaitlin, 1982), it was of interest to see whether IVR reduces the number of plasmodesmata in treated leaf tissues. In electron microscopy studies reported here no significant effect of IVR on plasmodesmata numbers, was observed, thereby negating this possibility.

The growing of Nicotiana tabacum L. Samsun NN plants and the preparation of protoplasts and IVR by precipitation with ZnAc_2 were as described before (Loebenstein & Gera, 1981). Control preparation were obtained similarly, from sham-inoculated protoplasts. Preparations obtained from 10^6 protoplasts, incubated for 72 h after inoculation, are termed one "unit" (Gera & Loebenstein, 1983).

Spray applications of IVR and control preparations (three units in 10 ml 0.05 M phosphate buffer, pH 7) were made to the upper side of the leaves of N. tabacum Samsun plants (2-3 weeks after transplanting) 5 h after inoculation with TMV (2.5 ug/ml). TMV was assayed after 72 h from the inoculated leaves - by homogenizing two 11-mm-diameter disks in 2 ml of 0.05 M phosphate buffer, pH 7.5. The homogenate was used to inoculate 12 half-leaves of N. glutinosa plants and compared with a standard solution of purified TMV on the opposite half leaf.

Three days after the spray applications, seven to ten leaf samples were cut from the lamina at the same positions from IVR and control-treated plants for electron microscopy. The samples were fixed for 1 hour in 2.5% glutaraldehyde in 0.02 M phosphate buffer pH 6.8, rinsed in the phosphate buffer, and additionally fixed for 1 hour in 1% osmium tetroxide in phosphate buffer. Dehydration was accomplished in 50%, 75% and 100% acetone. Infiltration and embedding were done with epoxy resin AGAR 100 (from Agar Aids). The epoxy blocks were cured over night at 70°C. Sections 200-250 nm thick were cut through the leaf parenchyma from the upper to the lower epidermis with the aid of an LKB Ultratome III, and were observed in the electron microscope without staining.

All plasmodesmata in one section between all palisade and spongy paranchyma cells were counted. Photographs of the same section were taken and the total length of common parenchyma cell walls was measured on the photographs, and the number of plasmodesmata per 100 um of common cell walls was calculated.

Results from three experiments comparing the effect of IVR and control preparations on virus titer and plasmodesmata number in Samsun leaves are summarized in Table 9. IVR significantly reduced virus titers, as measured by a reduction in extractable virus, by approximately 60%. No significant effect on number of plasmodesmata was observed. It seems, therefore, that the effect of IVR on virus restriction is not due to an effect on the number of plasmodesmata.

REFERENCES

- GERA, A. & LOEBENSTEIN, G. (1983). Further studies of an inhibitor of virus replication from tobacco mosaic-infected protoplasts of a local lesion responding tobacco cultivar. Phytopathology 73, 111-115.
- LEONARD, D.A. & ZAITLIN, M. (1982). A temperature-sensitive strain of tobacco mosaic virus defective in cell-to-cell movement generates an altered viral-coded protein. Virology 117, 416-424.
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- SHALLA, T.A., PETERSEN, L.J. & ZAITLIN, M. (1982). Restricted movement of a temperature-sensitive virus in tobacco leaves is associated with a reduction in numbers of plasmodesmata. Journal of General Virology 60, 355-358.

Table 9. Extractable infectivity and plasmodesmata number in Samsun tobacco plants sprayed with an inhibitor of virus replication (IVR, three units) or a control preparation 5 hours after inoculation.

Exp. No.	Treatment	Infectivity ^a	Inhibition of infectivity (%)	No. of plasmodesmata ^d
1	Healthy leaves			9.4 ± 0.9 ^e
	Leaves sprayed with control preparation ^b	53.6		11.4 ± 1.4 ^e
	Leaves sprayed with IVR ^c	19.5	64	8.2 ± 1.7 ^e
2	Leaves sprayed with control preparation ^b	50.0		11.8 ± 2.0 ^e
	Leaves sprayed with IVR ^c	17.6	65	13.1 ± 2.3 ^e
3	Healthy leaves			9.3 ± 1.1 ^e
	Leaves sprayed with control preparation ^b	43.6		9.4 ± 0.7 ^e
	Leaves sprayed with IVR ^c	18.5	58	8.6 ± 0.5 ^e

^a Average number of local lesions on one half-leaf of Nicotiana glutinosa, assayed 72 h after inoculation.

^b ZnAc₂ preparation from medium in which uninoculated protoplasts were suspended.

^c ZnAc₂ preparation from medium in which TMV-inoculated protoplasts were suspended.

^d Average number of plasmodesmata/100 um cell wall from 7-10 replicates. Means followed by the same letter are not significantly different (P<0/05).

2. Effect of IVR on protein synthesis in protoplasts. (in Israel)

To see if IVR affects general protein synthesis in protoplast, thereby also reducing virus, synthesis, ^{35}S -methionine incorporation was measured in IVR-treated Samsun and Samsun NN protoplasts. For controls non-treated protoplasts and protoplasts treated with sham-IVR were used. IVR (3 units) did not reduce ^{35}S -methionine incorporation neither into Samsun NN nor into Samsun protoplasts. On the contrary, a certain increase of incorporation was obtained, indicating perhaps that IVR inhibits virus replication via a cell mediated process (Table 10).

Table 10. Effect of IVR ^{35}S -methionine incorporation into acid insoluble fraction of protoplasts.

Protoplasts	72 hrs. Treatment	cpm/ug Protein
Samsun NN	IVR	75474
Samsun NN	mock IVR	50649
Samsun NN	control	59770
Samsun	IVR	75440
Samsun	mock IVR	56278
Samsun	control	50638

For each treatment 1×10^6 protoplasts were used. The results are averages of duplicates, which were treated with TCA, and counted twice. Protein determination was done twice and each experiment was repeated 3 times.

3. Effect of antimetabolites on IVR activity. (in Israel)

To see whether IVR acts directly on TMV replications or via another substance induced by IVR, the following preliminary experiments were performed.:

Actinomycin D 10ug/ml and chloramphenicol 200 ug/ml were mixed with IVR (3 unit) and added to 1×10^6 Samsun NN protoplast 5 hrs after inoculation with TMV. For controls the antimetabolites and IVR were added separately. Protoplasts were collected by centrifugation and homogenized. The homogenate was inoculated onto 12 half leaves on N. glutinosa plants and compared to control protoplasts without antimetabolites.

Table 11. Effect of Actinomycin D and Chloramphenicol on IVR activity^a

Treatment	Infectivity ^b from test protoplast incubated after inoculation for		Percentage of inhibition IVR/control	
	48 hr	72 hr	48 hr	72 hr
Control protoplasts	15.25	35.7		
IVR	4.3	10.5	62	61
Actinomycin D	28.6	54.2		
Chloramphenicol	31.5	55.1		
IVR + Actinomycin	13.7	50.4		
IVR + Chloramphenicol	12.9	51.6		

^aAverage of two to four experiments

^bAverage number of local lesions on one half-leaf on N. glutinosa

Table 11 shows that the addition of actinomycin D (an inhibitor of DNA-dependant - mRNA synthesis) and chloramphenicol (an inhibitor of protein synthesis) totally inhibits IVR activity. These results indicate that IVR acts via activators of the resistance mechanism in the protoplasts and not directly. These results will have to be verified by an additional approach, especially adding the antimetabolites at different times after inoculation.

H. Research on Preformed (Endogenous) Inhibitors of Virus Infection. (in US)

1. Inhibitor From Cotyledons and Mature Leaves of Cotton

a) Preparation

Cotton cotyledons (20-30 days old) or fully expanded cotton leaves were frozen and then homogenized 1:1 (w/v) with STANDARD BUFFER with a mortar and pestle. The homogenate was centrifuged at 2000g for 10 min and the supernatant collected, frozen, thawed and respun at 2000g for 10 min. This crude extract was used for further testing.

A portion of the extract was heated in a water bath to 55C for 10 min, spun at 2000g for 10 min and the supernatant collected for testing.

Extracts were mixed 1:1 (v/v) with TMV and inoculated onto one half of a Pinto Bean primary leaf. A Control solution of Buffer and TMV was inoculated onto the other half.

b) Results

Table 1. COTTON COTYLEDON EXTRACT (CEE)

Extraction Date	1 Exp.	Percent Inhibition (Lesion Number)	
		Untreated	55C-Treated
=====	=====	=====	=====
11/1/85	1	81.0	-9.0
	2	71.9	

11/4/85	1	75.3	47.5
	2	73.4	
	3	59.9	

1/13/86	1	75.6	33.3
	2	66.1	

1

Average of 8 Pinto Bean leaves per experiment

Table 2. COTTON LEAF EXTRACT (CLE)

Extraction Date	1 Exp.	Percent Inhibition (Lesion Number)	
		Untreated	55C-Treated
=====	=====	=====	=====
11/17/85	1	93.0	93.5
	2	97.5	93.0

1/9/86	1	57.7	
	2	53.3	55.8
	3	60.8	

¹
Average of 8 Pinto Bean leaves per experiment

c) Discussion

The inhibitor of virus establishment present in cotton cotyledons appears to be different than the inhibitor found in mature leaves, based on thermal sensitivity. A 10 min exposure to 55C reduces the inhibitory activity of CCF by about 50% while a similar heat treatment had no effect on the inhibitory activity of CLE. The potency of CLF apparently varies with the extraction possibly due to leaf position as the leaves extracted on 11/17/85 were from a mature (>6 months old) plant while the leaves extracted on 1/9/86 were the first three leaves formed.

II. Inhibitor from *Nicotiana glutinosa* leaves

a) Preparation

Fully expanded leaves of *Nicotiana glutinosa* were frozen and processed as for the cotton inhibitor (Section Ia).

b) Results

Assay Plant =====	Percent Inhibition (Lesion Number) =====	Number of leaves treated =====
<i>Datura stramonium</i>	62.5	6
<i>Phaseolus vulgaris</i> var. "Pinto"	60.5	10
<i>Nicotiana glutinosa</i>	44.4	10

c) Discussion

Although the results are preliminary, the inhibitor from *Nicotiana glutinosa* appears to be altruistic in that it does not inhibit virus (TMV) when tested on *N. glutinosa*.

Further testing is necessary before it can be said that the extract is actually stimulatory when mixed with virus and applied to its source plant.

d) References

Cheo, P.C. 1970. Subliminal Infection of Cotton by TMV. *Phytopathology* 60:41-46.

III. Datura EAVP

The inhibitor was obtained from frozen leaves of *Datura stramonium* after thawing and homogenizing in 0.067M phosphate buffer, pH 7.0. After discarding the pellet from a low speed centrifugation, the supernatant was concentrated by ammonium sulfate precipitation (61-80% saturation), dialyzed against buffer, filtered through Sephadex (G-75) and heated to 60C for 10 min.

Further concentration was done by molecular ultrafiltration producing the semi-purified Datura sap which was used for further tests.

A major reason for the work was to provide a basis for future research on the virus-induced inhibitor from Datura to simplify separation from the endogenous inhibitor.

Antiviral activity was monitored by the percent inhibition of lesion number produced when inhibitor and virus were mixed before inoculation.

A. Physical and Chemical Properties

1. Effects of Chemicals on Antiviral Activity

Approximately 45% of the inhibitory activity of DATURA EAVP was lost upon exposure to 5.5mM acetic anhydride (Table 1). No further reduction in antiviral activity was found after exposure to 55mM acetic anhydride. Exposure to phenyl isocyanate, which also attacks similar protein groups as acetic anhydride, also reduced DATURA EAVP antiviral significantly, by about 70% (Table 1).

Exposure to either concentration of performic acid or cysteine, both of which attack disulfide linkages, had no significant effect on antiviral activity of DATURA EAVP (Table 1).

Antiviral activity of DATURA EAVP was also not lost upon exposure to either concentration of formic acid (Table 1), which not only was used as a control for the performic acid experiments but also can attack specific peptide bonds.

Similarly treated control solutions of STANDARD BUFFER did not reduce the infectivity of TMV when mixed with virus and inoculated.

Antiviral activity of DATURA EAVP was not affected by the presence of EDTA (Table 2).

2. Effect of Enzymes

Inhibition of lesion number by DATURA EAVP was not significantly affected by the 2 hr exposure to papain or acid phosphatase (Table 3). Inhibitory activity was reduced by about 10% by exposure to trypsin (Table 3). Trypsin reduced the infectivity of TMV by 90%. Trypsin reduces the infectivity of TMV by precipitation. The presence of papain or acid phosphatase had no significant effect on the number of virus lesions produced by the STANDARD INOCULUM (Table 3: Row IIa vs. Row IIb, Row IVa vs. Row IVb). There was no evidence that DATURA EAVP experienced significant breakdown by incubation at 35C for 2 hrs (Table 3: Row IIIb vs. Row Vb).

3. Thermal Inactivation Point

The thermal inactivation point of DATURA EAVP is between 65C and 70C (Table 4). The antiviral activity of DATURA EAVP was not significantly reduced by incubation at 35C for 2 hrs or -15C for 2 years.

4. Effect of pH

The inhibitory extract - DATURA EAVP - has a pH of 6.9-7.0. The antiviral activity of DATURA EAVP is significantly reduced only by very low and high pH (Table 5). Significant reduction occurs between pH 2 and pH 3 but is not completely eliminated even at pH 1 (Table 5). However antiviral activity is completely eliminated at pH 11.

5. Proteinaceous Nature

The extract contained antiviral activity that correlated with A-280 absorbance and protein concentration. Precipitation by ammonium sulfate, inactivation at 65-70C, partial inactivation by trypsin and linear relation of antiviral activity with protein concentration leads to the conclusion that the substance isolated from Datura that is responsible for antiviral activity is a protein.

TABLE 1

Residual Antiviral Activity of DATURA EAVP Treated with
Chemical Reagents Which Affect Specific Protein Groups

Reagent	pH	Reaction Time (min)	Temp.	umoles/ml	Protein Groups	Residual Inhibitor Activity ¹
Phenyl isocyanate	8.1	15	25C	9.3	-NH ₂ , -OH	21.5, 37.7
Acetic anhydride	7.0	120	4C	5.5	-NH ₂ (alpha), -OH	63.4, 46.1
Acetic anhydride	7.0	120	4C	55.0	-NH ₂ (alpha & epsilon), -OH	68.4, 40.0
Formic acid	7.0	480	4C	0.44	Asp-Pro	99.4, 98.7
Formic acid	7.0	480	4C	4.4	Asp-Pro	98.4, 99.0
Performic acid	7.0	480	4C	0.44	-S-S-	98.0, 98.5
Performic acid	7.0	480	4C	4.4	-S-S-	96.0, 94.1
Cysteine	7.0	180	37C	25.0	-S-S-	93.0, 96.1

1

Each value represents percentage of inhibition of lesion number remaining as compared to an untreated DATURA EAVP+TMV Control. Concentration of DATURA EAVP was 50 ug/ml (Total Protein). All treated mixtures were dialyzed over-night against STANDARD BUFFER before assaying on 8 Pinto Bean half-leaves per experiment with similarly-treated STANDARD BUFFER used as a control.

TABLE 2

Effect of EDTA on Antiviral Activity when Mixed with DATURA EAVP.

Treatment	Average Lesion Number	Standard Deviation	Percent Inhibition
EDTA + TMV + STANDARD BUFFER	157.2 a	11.0	
EDTA + TMV + DATURA EAVP	6.3 *	2.4	96.0
STANDARD BUFFER + TMV	161.3 a	16.4	
DATURA EAVP + TMV	4.2 *	2.1	97.4

1

Concentration of EDTA was 1%. Values followed by the same symbol are not significantly different ($P < 0.01$) according to Student's t-test. A 1:1 (v/v) solution of EDTA and DATURA EAVP (50 ug/ml, Total Protein) was prepared and an equal volume of STANDARD INOCULUM was added before inoculating onto 6 Pinto Bean half-leaves. Control solutions were without DATURA EAVP and without EDTA.

TABLE 3

Effect of Enzymatic Cleavage on the Antiviral Activity of DATURA EAVP

Treatment	Replicate 1		Replicate 2		Percent Inhibition (11-c/b)x100)	
	1	Average Lesion Number	2	Average Lesion Number		
		S.D.		S.D.		
I. TRYPSIN [T] (1 mg/ml)						
a) Buffer + TMV (pH 8.0)		219.7	7.8			
b) T + TMV		19.20	5.8	20.10	2.9	
c) DATURA EAVP + T + TMV						
Expt. 1		2.2*	1.6	2.1*	1.9	88.5
Expt. 2		1.7*	2.0	1.0*	0.6	91.1
						95.0
II. PAPAIN [P] (1 mg/ml + 0.0005M cysteine)						
a) Buffer + TMV (pH 6.2)		210.6	18.9			
b) P + TMV		212.7	20.3	216.1	15.4	
c) DATURA EAVP + P + TMV						
Expt. 1		1.00*	0.82	0.87*	0.83	99.5
Expt. 2		0.57*	0.53	0.37*	0.52	99.7
						99.8
III. DATURA EAVP CONTROLS for I and II						
a) Buffer + TMV (pH 7.0)		213.7	20.7			
b) DATURA EAVP + TMV						
Expt. 1		0.830	0.98	0.00	0.0	99.6
Expt. 2		0.370	0.52	0.630	0.74	99.8
						100
						99.7

TABLE 3 (cont.)

Effect of Enzymatic Cleavage on the Antiviral Activity of DATURA EAVP

Treatment ¹	Replicate 1		Replicate 2		Percent Inhibition (11-c/b)x100)	
	Average Lesion Number	S.D. ²	Average Lesion Number	S.D.		
-----	-----	----	-----	----	-----	-----
IV. ACID PHOSPHATASE [PH] (2 units/ml)						
a) Buffer + TMV (pH 5.0)	99.6	11.8				
b) PH + TMV	95.0	16.5	83.4	9.5		
c) DATURA EAVP + PH + TMV	7.0*	3.3	6.8*	2.8	92.6	91.8
V. DATURA EAVP CONTROLS for IV						
a) Buffer + TMV (pH 7.0)	88.4	9.4				
b) DATURA EAVP + TMV	1.62	1.5	5.00	4.3	98.4	94.0

¹ Each value represents the average of treatments applied to 6-8 Pinto Bean half-leaves. Enzyme solutions were made up in 0.067M Phosphate buffer at the optimum pH for that enzyme. Solutions were mixed with STANDARD BUFFER or DATURA EAVP, the pH was readjusted and the mixtures were incubated at 35C for 2 hours.

² S.D. - Standard Deviation

Symbols

- ® - Significantly different ($P < 0.005$) from a) according to Student's t-test.
- * - Significantly different ($P < 0.005$) from b) according to Student's t-test.

TABLE 4

Thermal Inactivation Point of DATURA EAVP

1 Temperature -----	Lesion Number- Treated -----	2 S.D. -----	Lesion Number- Control -----	S.D. -----	Percent Inhibition -----
Room (22C)	3.7 *	2.9	135.0	22.0	97.3
60C	0.43 *	0.7	34.3	9.5	98.7
65C	18.3 *	5.3	117.6	18.6	84.5
70C	226.0	17.0	223.0	16.8	-1.3
75C	236.5	29.0	223.0	16.8	-6.0
80C	40.7	18.2	41.3	8.7	1.4

1
STANDARD BUFFER (Control) and DATURA EAVP (50 µg/ml Total Protein) solutions were raised to the desired temperature and then exposed for 10 minutes. Solutions were cooled with cold tap water to room temperature and centrifuged at 20000 for 10 minutes before mixing with STANDARD INOCULUM and assaying on 8 Pinto Bean half-leaves per experiment.

2
Standard Deviation

Symbols: Significantly different ($P < 0.005$) (*) from Control according to Student's t-test.

TABLE 5

Effect of pH on the Antiviral Activity of DATURA EAVP

1 pH -----	Expt. Num. -----	Lesion Number- Treated -----	2 S.E. -----	Lesion Number- Control -----	S.E. -----	Percent Inhibition -----
1	1	129.0*	17.0	166.0	13.0	22.3
	2	116.0\$	15.4	145.0	33.0	20.0
2	1	18.10	7.5	25.7	11.3	29.6
	2	21.6	6.5	25.7	11.3	16.0
3	1	8.7*	5.1	43.3	9.9	79.9
	2	6.1*	3.5	39.1	11.0	84.0
4	1	13.5*	6.8	88.0	13.4	84.7
	2	11.4*	4.7	88.0	13.4	87.0
6	1	5.3*	3.2	88.0	13.4	94.0
	2	7.3*	5.2	88.0	13.4	91.7
7	1	3.5*	3.4	55.0	9.4	93.6
	2	6.7*	2.6	305.0	137.0	97.8

TABLE 5 (cont.)

Effect of pH on the Antiviral Activity of DATURA FAVP

1 pH	Expt. Num.	Lesion Number- Treated	2 S.D.	Lesion Number- Control	S.D.	Percent Inhibition
-----	-----	-----	-----	-----	-----	-----
8	1	11.4*	5.7	88.0	13.4	87.0
	2	12.0*	5.8	88.0	13.4	86.4
9	1	1.7*	1.1	20.4	9.7	91.8
	2	1.4*	0.8	18.0	3.4	92.2
10	1	7.7*	3.0	43.6	9.3	82.2
	2	8.7*	5.1	41.3	8.7	78.9
11	1	85.3	26.7	88.0	13.4	3.1
	2	121.0	18.0	118.0	14.0	-2.0

1

The pH of STANDARD BUFFER and DATURA FAVP solutions was changed by dropwise addition of 1N HCl or 1N NaOH. After one minute, the pH was brought back to pH 7 by the addition of 1N NaOH or 1N HCl. The solutions were then centrifuged at 2000g for 10 min., the supernatant mixed with STANDARD INOCULUM and assayed on 8 Pinto Bean half-leaves per experiment.

2

S.D. - Standard Deviation

Symbols

- * - Significantly different ($P < 0.005$) from Control according to Student's t-test.
- s - Significantly different ($P < 0.10$) from Control according to Student's t-test.
- @ - Significantly different ($P < 0.20$) from Control according to Student's t-test.

II. Biological Properties

1. Systemic Effects

The number of local lesions on the upper surface of Pinto Bean leaves inoculated with TMV was not reduced when DATURA EAVP was applied to the underside of the same leaf up to 1 day before inoculation (Table 6).

However, lesion numbers were significantly reduced when inhibitor was applied to the upper surface even 5 days before inoculation of the same surface.

Untreated half-leaves demonstrated no acquired resistance even 7 days after application of the adjacent half with DATURA EAVP (Table 6).

2. Influence of Identity of Virus

All viruses tested contained positive ("+")-sense ssRNA (Table 7). When mixed with inhibitor, lesion numbers of TMV were reduced by 68% but AMV was not affected. Members of the PVX group gave variable results - PVX was inhibited about 50% while CaVX was not affected.

The variable results encountered when members of the PVX group were tested implies that the assay host has at least two types of virus receptors, only one of which was inhibited by DATURA EAVP.

3. Influence of Taxonomic Status of Assay Host

The inhibitor showed >90% activity when assayed on plants from the Fabaceae and Chenopodiaceae (Table 8).

However, no activity was manifest when assayed on the source host - *Datura stramonium*.

Other members of the Solanaceae produced variable results. Activity was greater than 90% on *Nicotiana glauca* but less than 65% on several varieties of *N. tabacum*. Activity was still over 40% when tested on a member of the same genus - *Datura metel*.

Therefore, the relation between the degree of inhibition and the taxonomic status of the assay host with respect to *Datura stramonium* is determined at the species level.

4. Effect on Viral Replication

Employing two varieties of tobacco (Xanthi and Samsun) which give a systemic infection with TMV, virus titers were not significantly reduced 12 days after inoculation with virus-inhibitor mixtures (Figures 1 and 2). In both varieties there was a lag period in virus increase between days 4 and 5 induced by the inhibitor; after day 5 there was no such effect.

5. Mechanism of Inhibition

Van Kammen, et al (Virology 14:100-108, 1961) suggest that virus and host-cell receptor react reversibly. The authors derived an equation that resembled a Lineweaver-Burk plot of enzyme kinetics:

$$E/e = 1 + K(v)/c$$

where E = maximum number of lesions
 e = number of lesions at virus concentration c
 $K(v)$ = dissociation constant of virus-receptor complex

When they analyzed data resulting from the use of an inhibitor from carnation, they concluded that the inhibitor was acting competitively with the virus.

We made a similar analysis using DATURA EAVP and came to a similar conclusion (Figure 3). Competitive inhibition was also indicated from plots of percent inhibition vs. inhibitor concentration at constant virus concentration (Figure 4).

TABLE 6
SYSTEMIC ANTIVIRAL ACTIVITY OF DATURA EAVP

I. Application of DATURA EAVP onto the underside of the leaf.

A. Effect on Lesion Number

1 Day	Expt. Num.	Average Lesion Number- Treated	2 S.D.	Average Lesion Number- Control	2 S.D.	Percent Inhibition
---	---	-----	----	-----	----	-----
0	1	735	104	650	82	-13
	2	613	78	605	75	-1
1	1	206	42	225	73	8.4
	2	306	41	310	39	1.3

B. Effect on Lesion Diameter

Day	Expt. Num.	Average Lesion Diameter- Treated	S.D.	Average Lesion Diameter- Control	S.D.	Percent Inhibition
---	---	-----	----	-----	----	-----
0	1	.260mm(50)	.11	.250mm	.14	-4.0
	2	.257mm(50)	.09	.262mm	.12	-2.0
1	1	.287mm(50)	.12	.294mm	.13	2.0
	2	.285mm(50)	.10	.280mm	.12	-2.0

1
DATURA EAVP (50 ug/ml Total Protein) was applied to the underside of 8 Pinto Bean leaves per experiment 0 or 1 days before TMV inoculation of the upper side. STANDARD BUFFER applied to the underside was treated similarly and used as a control.

2
Standard Deviation

3
Number of lesions measured for both Control and Treated leaves.

TABLE 6 (continued)

Comparison with Systemic Acquired Resistance (SAR) Induced by TMV.						
1	Average	2	Percent	Average		Percent
Inducer	Lesion	S.D.	Inhibition	Lesion	S.D.	Inhibition
-----	Number	-----	-----	Diameter	-----	-----
DATURA				3		
EAVP	158	49	-1	.38mm(75)	.12	-4
TMV	20	12	87 *	.33mm(55)	.10	9.6 **
Buffer						
Control	156	54		.36mm(65)	.14	

1
Eight Pinto Bean plants were used per treatment. Half leaves were inoculated with either STANDARD BUFFER, DATURA EAVP, or STANDARD INOCULUM. Seven days later, the adjacent half leaf was inoculated with TMV and lesions were assayed 6 days later.

2
Standard Deviation

3
Number of lesions measured.

Symbols: Significant at ($P < 0.005$) (*) and ($P < 0.20$) (**) compared with Buffer Control according to Student's t-test.

TABLE 7

Effect of Virus Particle Morphology on
the Antiviral Activity of DATURA EAVP

1 Plant Virus Group	2 Lesion Number- Control	3 S.D.	Lesion Number- DATURA EAVP	S.D.	Percent Inhibition
-----	-----	-----	-----	-----	-----
ALFALFA MOSAIC VIRUS GROUP (bacilliform, tripartite)					
Alfalfa Mosaic Virus	44.4	16.8	47.1	10.6	-6.1
TOBACCO NECROSIS VIRUS GROUP (isometric, monopartite)					
Tobacco Necrosis Virus	28.7	13.4	9.2 *	7.7	68.0
POTEXVIRUS GROUP (flexuous rods, monopartite)					
Cactus Virus X	54.1	18.8	52.2	20.7	3.5
Potato Virus X					
Expt.1	986.0	82.0	314.0 *	39.0	64.6
Expt.2	105.0	21.2	61.1 *	23.0	42.0
TOBAMOVIRUS GROUP (rigid rods, monopartite)					
Tobacco Mosaic Virus					
Expt.1	262.0	25.0	24.0 *	6.5	90.8
Expt.2	319.0	49.0	8.1 *	2.3	97.0
Expt.3	350.0	38.0	7.0 *	1.7	98.0
Ribgrass Mosaic Virus	16.4	7.5	2.6 *	1.7	84.0

1 All viruses contain "+" ssRNA.

2 Assayed on 8 half-leaves of *Chenopodium amaranticolor* per experiment. Virus was mixed with DATURA EAVP (50 ug/ml Total-Protein) or STANDARD BUFFER and inoculated. Lesions were counted 6-8 days later.

3 Standard Deviation
Symbols: Significant at ($P < 0.005$) (*) compared with Control lesion number according to Student's t-test.

TABLE 8

Relation of the Systematic Position of the Test Plant
on the Antiviral Activity of DATURA EAVP Against TMV

Plant Species =====	Percent Inhibition (Lesion Number) =====
ASTERIDAE	
Solanaceae	
Nicotiana tabacum -----	
var. Xanthi-nc	62.4
Nicotiana tabacum -----	
var. Samsun NN	64.5
Nicotiana glutinosa -----	95.7, 94.3, 97.1
Datura stramonium -----	-4.0, 1.5
Datura metel -----	44.0
ROSIDAE	
Fabaceae	
Phaseolus vulgaris -----	
var. "Pinto"	97.4, 99.0, 93.6
CARYOPHYLLIDAE	
Chenopodiaceae	
Chenopodium amaranticolor -----	97.5, 98.0

¹ Each value represents results from one experiment. STANDARD INOCULUM and DATURA EAVP (50 ug/ml, Total Protein) were mixed and applied to 6-8 half-leaves per experiment. A mixture of STANDARD INOCULUM and STANDARD BUFFER was applied to the adjacent half-leaf as a control.

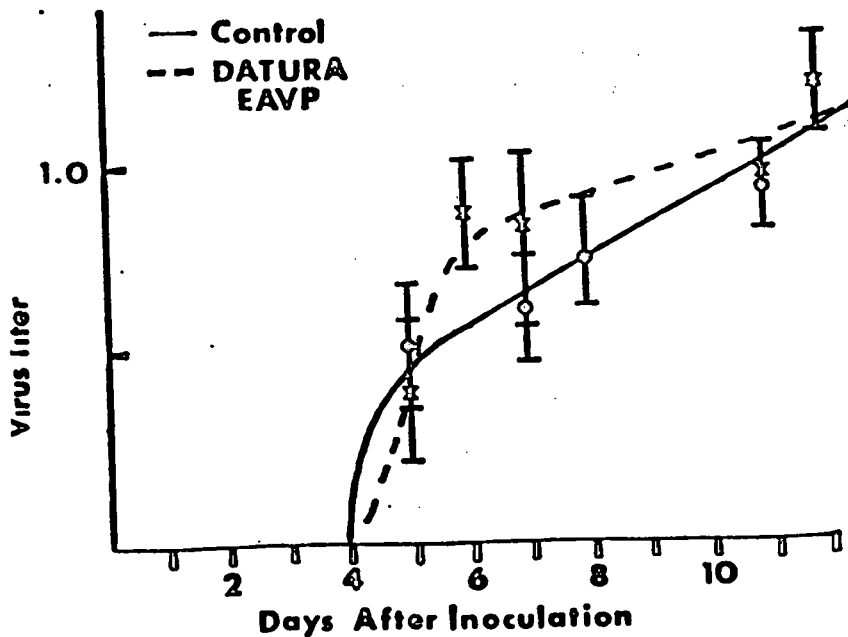


Figure 1. Effect of DATURA EAVP mixed with TMV on virus titer on *Nicotiana tabacum* var. Xanthi. Virus titer is expressed as the ratio of average lesion number to average lesion number produced by the STANDARD INOCULUM on Pinto Bean half-leaves. Error bars represent one standard deviation, means are the results of 2 experiments, 2 replicates for days 5-9. Xanthi leaves were inoculated with DATURA EAVP (50 ug/ml Total Protein) mixed with STANDARD INOCULUM. TMV mixed with STANDARD BUFFER was inoculated onto separate Xanthi plants as a control. Six Xanthi leaves were collected per day. A clarified extract was inoculated onto 8 Pinto Bean half-leaves. The adjacent half was inoculated with STANDARD INOCULUM.

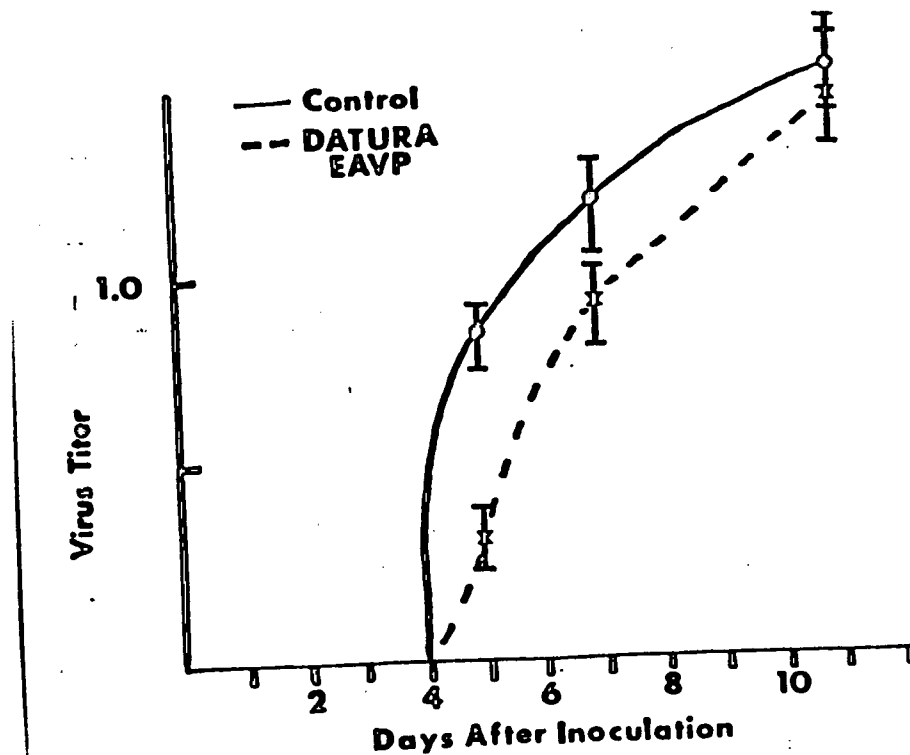


Figure 2. Effect of DATURA EAVP mixed with FMV on virus titer on *Nicotiana tabacum* var. Samsun. Virus titer is expressed as the ratio of average lesion number to average lesion number produced by the STANDARD INOCULUM on Pinto Bean half-leaves. Error bars represent one standard deviation, means are the results of 2 experiments, 2 replicates for days 4-9. Samsun leaves were inoculated with DATURA EAVP (50 ug/ml Total Protein) mixed with STANDARD INOCULUM. FMV mixed with STANDARD BUFFER was inoculated onto separate Samsun plants as a control. Six Samsun leaves were collected per day. A clarified extract was inoculated onto 8 Pinto Bean half-leaves. The adjacent half was inoculated with STANDARD INOCULUM.

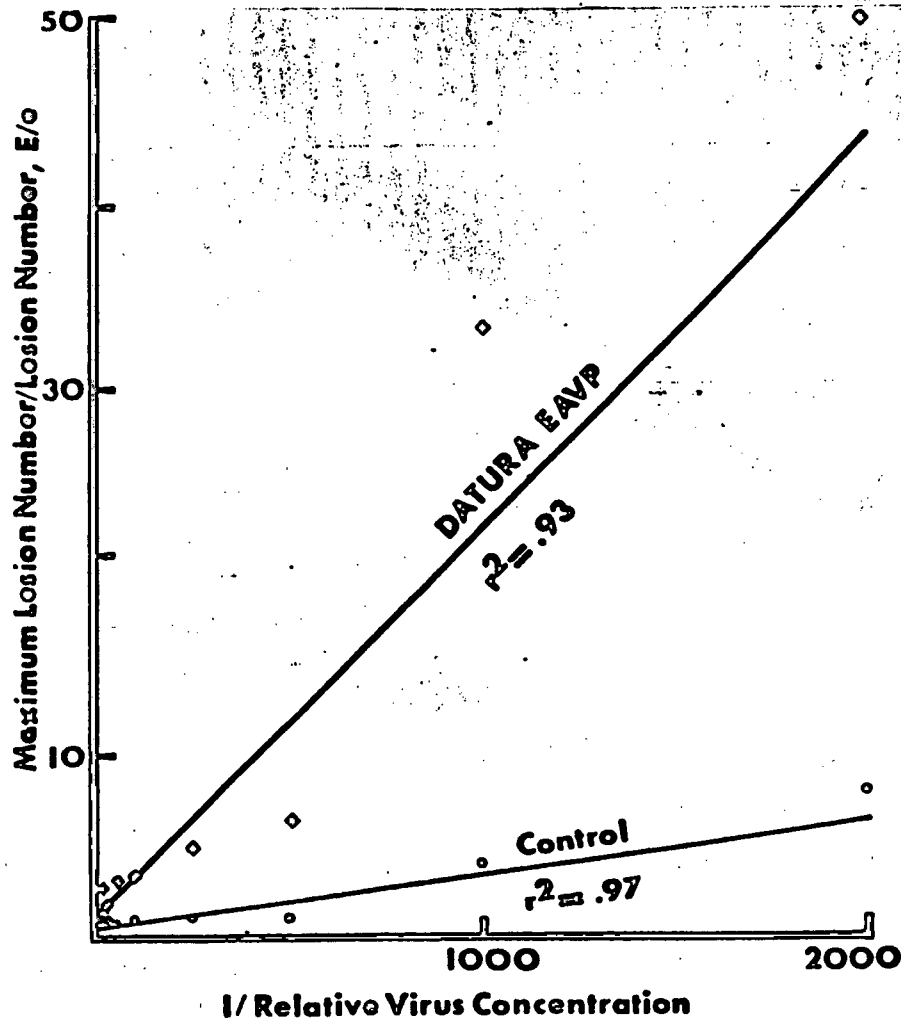


Figure 3. The relation between 1/relative virus concentration and the ratio of the maximum number of local lesions (E) and the number of local lesions (e) when virus is mixed with STANDARD BUFFER (Control) or DATURA EAVP (50 ug/ml Total Protein). Each point represents the mean obtained from treatments applied to 8 Pinto Bean half-leaves. The y-intercepts of both lines are not significantly different from each other ($P < 0.005$) as determined by the Student's t-test.

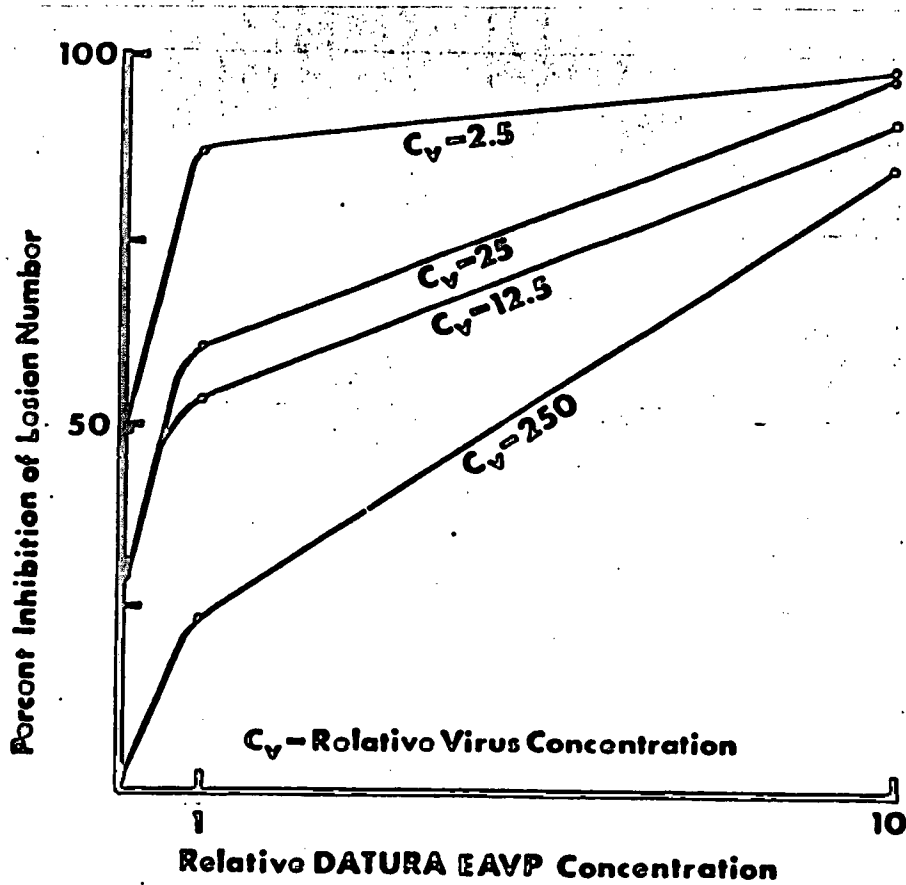


Figure 4. The effect of increasing relative virus concentration on the relationship between the percent inhibition of lesion number at various relative concentrations of DATURA EAVP. Each point represents the average obtained from treatments applied to 8 Pinto Bean half-leaves.

I. Ultraviolet Photobiology of Virus-Host Interaction (in US).

I. Effect of UV-C Irradiation on TMV-Induced Green Islands in Systemic Tobacco Leaves

a) Methods

Two lower leaves of *Nicotiana tabacum* var. Wisc. 38 (2 months old) were inoculated with TMV (20 ug/ml) and kept in the growth chamber until green islands were developed in upper leaves which were then exposed to various doses of UV-C (85% at 253.7 nm) followed by 24 hrs dark treatment to prevent photoreactivation. Seven days after irradiation, leaves were harvested, green island tissue and nongreen tissue were separated and clarified extracts were used to determine virus titer by ELISA.

b) Results

The green island tissues had less virus than nongreen tissues as can be seen in Table 1. UV-C irradiation increased virus titer in green island tissues presumably by breaking down the resistance mechanism. A UV-C dose of 37.44 ergs per square centimeter was the optimum dose to suppress the resistance mechanism; both higher and lower doses were less effective. The decrease of virus titer at higher doses was probably due to a combination of damage to host metabolism and direct virus inactivation. On the other hand, as can also be seen in Table 1, UV-C tended to decrease virus titer in the nongreen tissue.

II. Effect of UV-C Irradiation on Localized Acquired Resistance

a) Samsun NN

Samsun NN tobacco mid-leaves were rubbed with a dilute suspension of TMV giving approximately 10 local lesions per half-leaf. Twenty four hours later, inoculated leaves were exposed to the UV-C doses listed in Table 2. Seven days later, leaves given the primary rub as well as controls which had not, were challenged with a high concentration of TMV. Controls were included to assess the effects of UV-C on subsequent infection to virus.

One week later, numbers and sizes of lesions were determined on the singly-infected leaves while areas of resistant zones (excluding the primary lesion itself) were measured around the primary lesions and averaged.

As can be seen, the size of resistant zones increased with increasing dose of UV-C, and while there was some inhibition of lesion numbers and size that could be attributed to pre-inoculation irradiation, this could not account for the magnitude of the UV-C effect in decreasing the area of challenge lesion exclusion around the primary lesions.

b) Pinto Bean

Pinto Beans were tested in a similar fashion, except that virus was rubbed initially at high concentration in alternating strips separated from non-inoculated areas. Ultraviolet treatment was as noted with Samsun NN tobacco, and entire leaves were challenged one week after the primary inoculation with the same inoculum. We noted how closely challenge lesions came to the primary lesions. In this case, UV-C also partially overcame localized acquired resistance, but not to the extent shown in the experiments with tobacco.

III. Effect of UV-C on Localization

Fully mature leaves of 2-month old plants of *Nicotiana glutinosa* and the primary leaves of 10-12 day old Pinto Bean plants were used in these experiments. Leaves were rubbed with a concentration of TMV giving 100-200 lesions per leaf.

Leaves were exposed to the doses of UV-C shown in Figures 1 and 2 24 hrs after inoculation, placed in the dark for 24 hrs before being returned to the greenhouse and lesion numbers and areas measured one week after inoculation.

As shown in the earlier experiments, UV-C caused delocalization in bean, with respect to increases in lesion numbers and areas. In contrast, decreases in both lesion numbers and areas were observed in *N. glutinosa*. This difference is puzzling but may be related to the fact that where UV-induced delocalization has been noted by Loebenstein, it tended to be at a lower level than that shown by other species.

Table 1
Effects of Ultraviolet irradiation on TMV-induced Green Islands in Tobacco Leaves

UV Dose (ergs X 10 ⁴)	(C)		(T)		(C)		(T)	
	Green Island	UV Green Island	Green Island	UV Green Island	Virus Titer (ug ml ⁻¹) T/C Nongreen Tissue	UV Nongreen Tissue	Virus Titer (ug ml ⁻¹) T/C Nongreen Tissue	UV Nongreen Tissue
2.08	40.53	43.33	1.07	63.36	65.65	1.04	65.65	1.04
4.16	42.00	57.77	1.38	62.35	57.93	0.93	57.93	0.93
8.32	53.33	61.52	1.15	62.28	56.47	0.91	56.47	0.91
12.48	49.98	59.85	1.20	58.27	50.34	0.86	50.34	0.86
24.96	46.44	57.32	1.23	76.90	55.44	0.72	55.44	0.72
37.44	56.64	96.80	1.71	77.54	53.10	0.68	53.10	0.68
49.92	53.77	66.24	1.23	68.81	41.77	0.60	41.77	0.60
62.40	58.80	51.31	0.87	69.96	40.23	0.58	40.23	0.58

Table 2. Effect of UV-C Irradiation on
Localized Acquired Resistance.

UV-C Dose -4 (ergs x10)	Lesion Free Area Ratio	1
=====	=====	
	Samsun NN	

12.48		0.51
24.96		0.36
37.44		0.22
49.92		0.09
	Pinto Bean	

6.24		0.60
12.48		0.50

¹
Average lesion-free area around primary lesion (excluding primary lesion area) from irradiated tissue divided by the comparable area from control tissue.

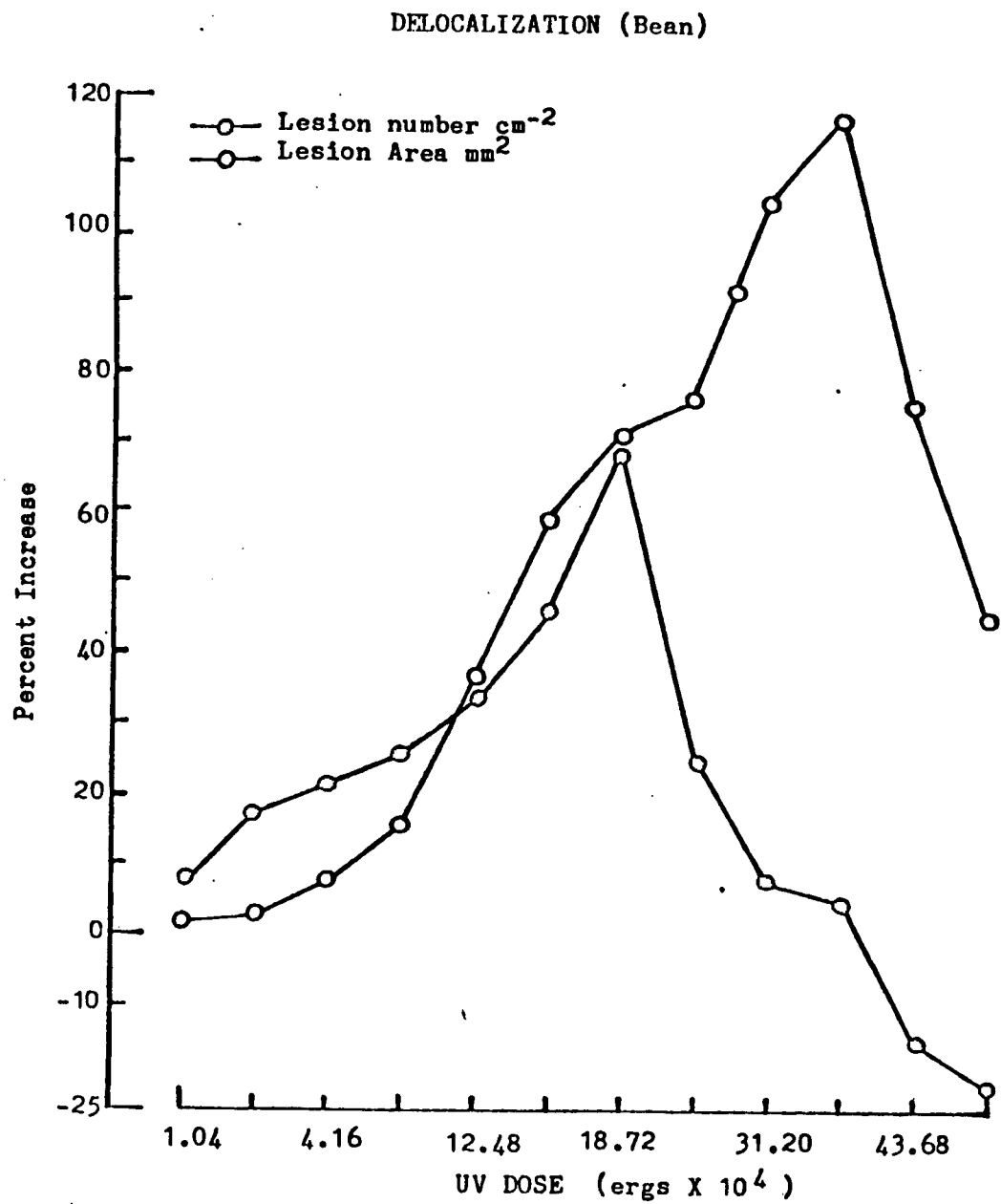


Figure 1. Effect of Post-inoculation UV-C Irradiation on Lesion Number and Area of TMV-infected Pinto Bean Leaves.

DELOCALIZATION (N. Glutinosa)

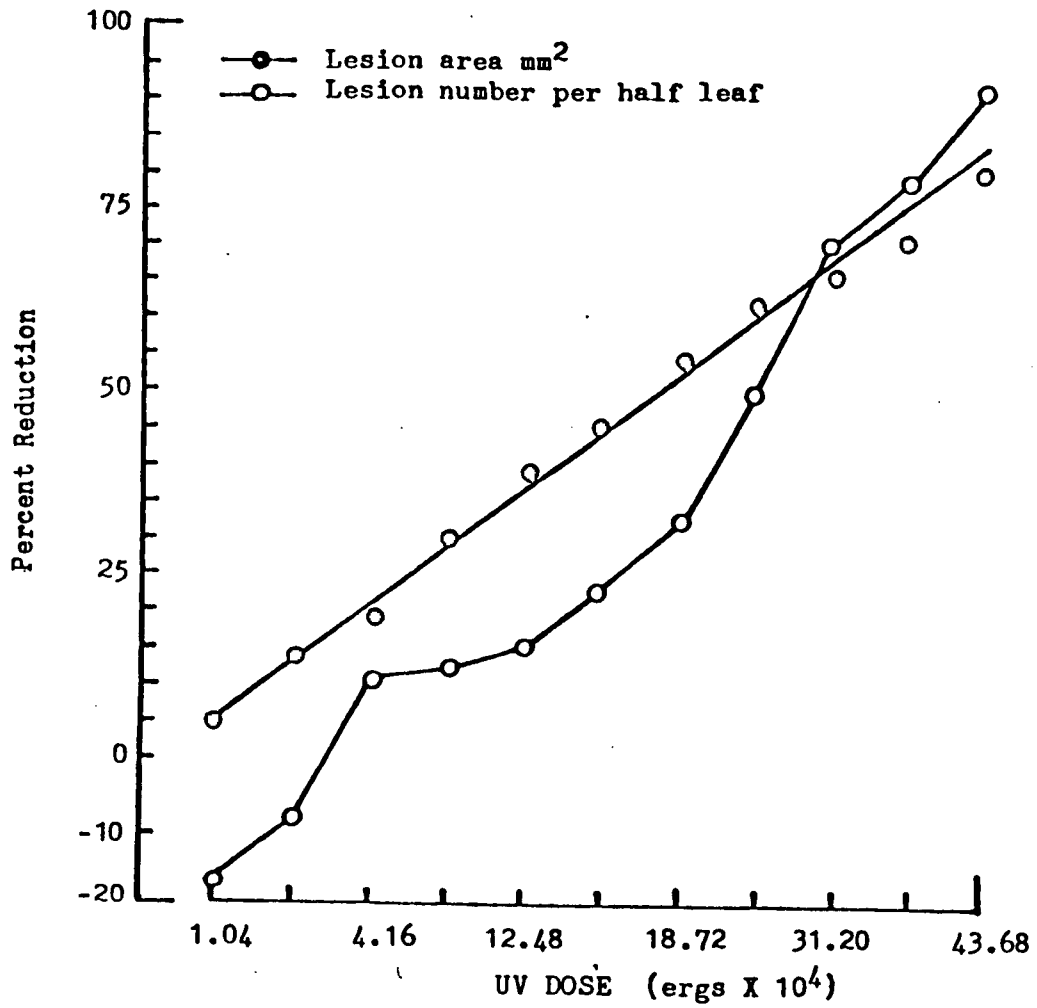


Figure 2. Effect of Post-inoculation UV-C Irradiation on Lesion Number and Area of TMV-infected Nicotiana glutinosa Leaves

IV. Effect of UV-C on Leaf Capacity

Ultraviolet light has long been known to generally produce a decrease in lesion numbers upon subsequent inoculation with virus. It has been assumed that only establishment was involved. We have extended this work to include measurements of lesion area as an indicator of effects on viral replication.

Using the same two hosts and the same general experimental conditions, we irradiated leaves of both species with the UV-C doses shown in Figures 3 and 4, placed the plants in the dark for 24 hrs, then inoculated them with TMV and placed them in the greenhouse. Seven days after inoculation, both lesion numbers and areas were determined.

In agreement with previous work, lesion numbers decreased with increasing dose for both species. Similar results were obtained for lesion area in bean, although not to the same extent as numbers. At the lowest dose employed, *M. glutinosa* already showed a marked reduction in lesion area but, this effect changed relatively little as doses were increased.

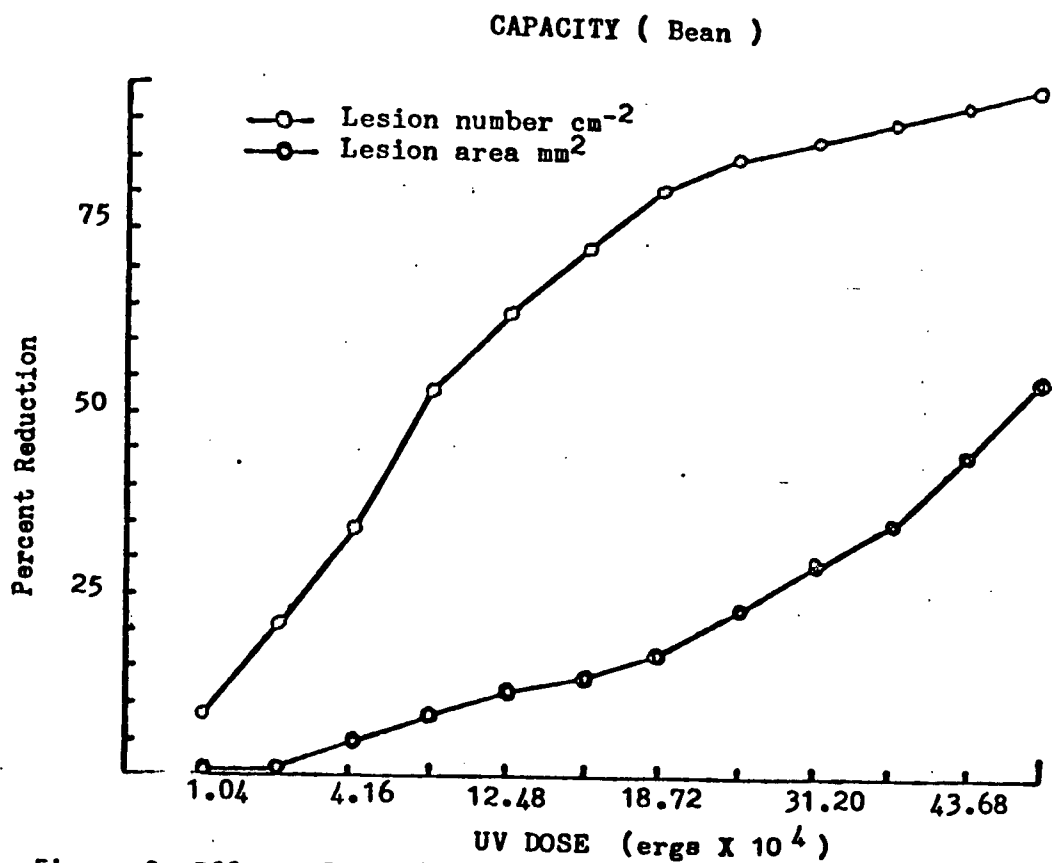


Figure 3. Effect of Pre-inoculation UV-C Irradiation on Lesion Number and Area of TMV-infected Pinto Bean Leaves

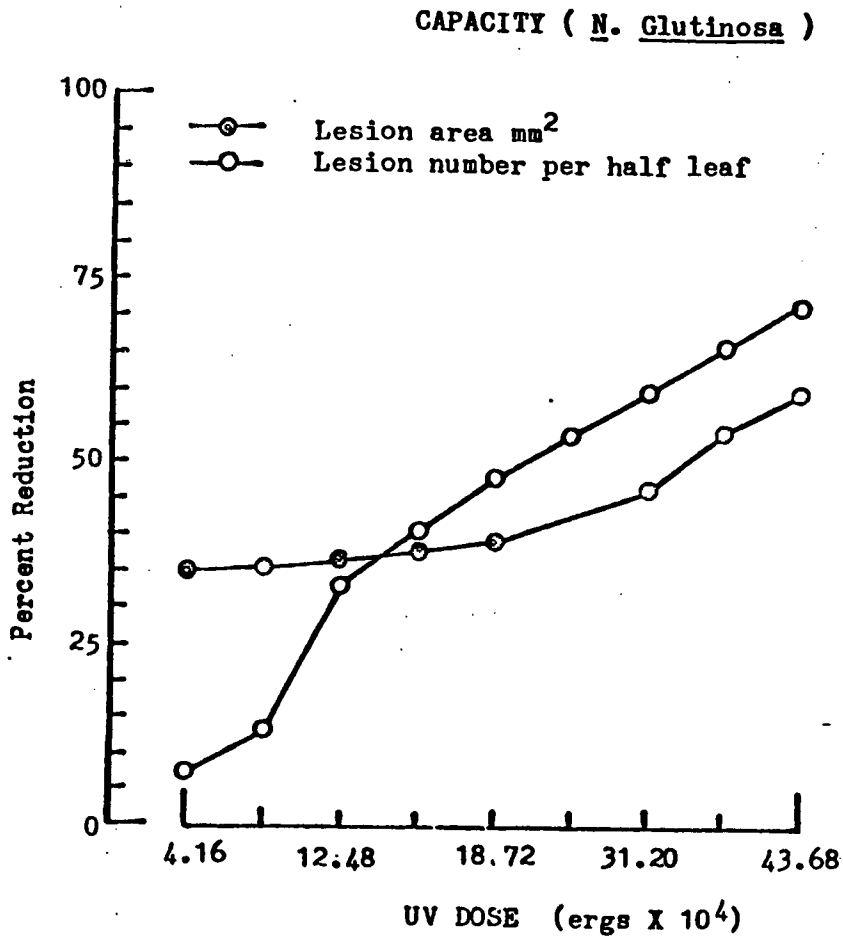


Figure 4. Effect of Pre-inoculation UV-C Irradiation on Lesion Number and Area of TMV-infected Nicotiana glutinosa Leaves.

f. Description of cooperation

During the course of the present grant period, exchange visits were made. M. Chessin visited the Volcani Center in the Spring of 1985, during which time discussions were held and several seminars presented. A brief visit to Dr. Sela's lab was also made during that time.

In August, 1986, Dr. A. Gera spent several days in Missoula and had fruitful discussions with our research staff.

As part of the cooperation parallel research on the protoplast system were conducted, resulting in the Ph.D. thesis of A. Mitra which corroborates many of the results obtained in Israel.

g. Evaluation of the research achievements

Overall the project progressed as planned. Methods for preparation of IVR were improved markedly and IVR-like compounds were obtained from "green island" tissue as well as from the intercellular fluid of Samsun NN tobacco and Pinto beans inoculated with TMV. Antisera to the two fractions of IVR were prepared and used in further purification of IVR. Induction of IVR in protoplasts was studied parallel to the development of resistance. A "unit" of activity was defined, and studies on possible modes of action of IVR were started.

IVR was evaluated on two additional infected intact plants, though the amounts of IVR required for such studies did not enable us to intensify this area of research.

In Missoula emphasis was placed on the use of the protoplast system with TMV and *Nicotiana* spp., instead of testing new host virus combinations for acquired resistance.

Both a virus-induced and an endogenous inhibitor were characterized (58,000d and 24,500d, respectively). The induced inhibitor is probably a phosphorylated glycoprotein with its antiviral activity due to the inhibition of viral-RNA-directed protein synthesis.

Work with preformed inhibitors emphasized a more complete physico-chemical and biological characterization of the *Datura* inhibitor. The latter acts by binding competitively to cell receptors and by altering the affinity of such receptors for virus.

Experiments were done on the characterization of the endogenous inhibitor of cotton, which was the classical host for subliminal infection by TMV, as well as on an endogenous inhibitor from *Nicotiana glutinosa*. We obtained evidence that there are 2 such inhibitors of virus establishment in cotton.

Work on photobiology of the viral infection continued. Additional data on the phenomenon of photolocalization; i.e., a previously noted

effect of UV-C and visible light after inoculation with virus in producing smaller lesions than those produced by visible light alone, were obtained.

No such consistent effects were observed. Unlike Pinto Bean, tobacco continued to show the failure of post-inoculation UV-C to produce delocalization (larger lesions) of TMV. However, delocalization in tobacco was possible with post-inoculation darkness or high temperature. This distinction may be helpful in determining the detailed mechanism of virus-induced resistance.

Another unexpected finding was the decreased lesion size resulting from pre-inoculation irradiation, implying an effect on the viral replication mechanism. In line with this, we noted a generally decreasing virus titer with increasing doses of UV-C given before inoculation.

UV-C increased virus titer in "green islands" resulting from TMV infection in systemic tobacco. This agrees with the concept that "green islands" are another manifestation of induced resistance. An inhibitor of viral replication has been isolated from such tissues in Prof. Loebenstein's laboratory.

We also obtained some evidence that UV-C could partially suppress localized acquired resistance as it also does for localization. This suggests that localization and LAR are closely related phenomena. On

the other hand, we have shown previously that systemic acquired resistance did not respond to UV-C treatment.

The research has reached a stage now, after obtaining IVR in larger quantities, its additional purification, evidence that IVR is a good antigen and preliminary purification by HPLC, that monoclonals can be prepared and used for obtaining m-RNA; or alternatively amino acid analysis of sequences of IVR after HPLC, for synthesizing a c-DNA probe can be approached. This should lead to the isolation and cloning of the IVR gene and its introduction into susceptible plants in the future.

List of Publications

- [1] Chessin, M. (1983): Is there a plant interferon?. Bot. Review 49: 1-23.
- [2] Chessin, M. (1984): Antiviral proteins in plants. NWSA-MAS Conf. Missoula, Montana, (abstr.).
- [3] Chessin, M. and Mitra, A. (1985): Effects of UV irradiation on infection with tobacco mosaic virus. Phytopathology 75: 1292 (abstr.).
- [4] Gera, A., Loebenstein, G. and Shabtai, S. (1983): Enhanced tobacco mosaic virus production and suppressed synthesis of a virus inhibitor in protoplasts exposed to antibiotics. Virology 127: 475-478.
- [5] Gera, A., Spiegel, S. and Loebenstein, G. (1986): Production, preparation and assay of an antiviral substance from plant cells. In: Methods in Enzymology, 119: 729-734.
- [6] Geske, S.M. and Chessin, M. (1986): Characterization of intercellular wash fluid (IWF) from tobacco mosaic virus (TMV) infected Pinto bean plants. Phytopathology 76: 1062 (abstr.).
- [7] Loebenstein, G., Gera, A. and Stein, A. (1984): Plant defense mechanisms against viral infections. In "Control of Virus Diseases". pp. 375-391 Editor: E. Kurstak. Marcell Dekker, Inc., New York.
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- [10] Stein, A., Spiegel, S. and Loebenstein, G. (1985): Studies on induced resistance for tobacco mosaic virus in Samsun NN tobacco and changes in ribosomal fractions. Phytopath. Z. 114: 295-300.
- [11] Zipf, A. and Chessin, M. (1985): Characterization of an endogenous proteinaceous inhibitor of plant virus from Datura stramonium. Phytopathology 75: 1293 (abstr.).